Remarks and Response to Arguments

Claims 1-20 are pending. Claims 6-16 have been withdrawn. Support for newly added claims 19 and 20 can be found throughout the application, and specifically on page 19, lines 21-22, page 21, lines 15-19, and page 22, lines 2-5. No new matter is believed to be added by these amendments.

Rejection under 35 U.S.C. § 102

Claims 1-4 and 17-18 are rejected under 35 U.S.C. 102(b) as allegedly being anticipated by Farrington et al. The Examiner states that Farrington et al. discloses a method for detecting soluble antigen using monoclonal antibodies against *C. parvum* oocysts.

This rejection is respectfully traversed. Claims 1-4 and 17-18 are drawn to a composition comprising an antibody specific for a soluble antigen of a *C. parvum* sporozoite. Farrington et al. discloses an "Anti-*Cryptosporidium* oocyst monoclonal antibody". The term "specific for" applies to each element listed: soluble, *C. parvum*, and sporozoite. This is the accepted meaning of "specific" in the art, as discussed below. However, the antibody of Farrington et al. is neither specific for *C. parvum*, as it is described as a *Cryptosporidium* oocyst monoclonal antibody and not just as a *C. parvum* antibody (page 9, 2nd paragraph), nor is the antigen soluble, as Farrington et al. states "Soluble *or finely particulate* antigen was detected...using the MAb C1" (page 14, first full paragraph, emphasis added.) Therefore, since Farrington et al. were not even sure if they were detecting soluble antigens or finely particulate antigen, and since there is no evidence to show detection of soluble antigens, this reference cannot be relied upon as prior art. Furthermore,

since the assay was capable of detecting both soluble and finely particulate antigen, the antibody is not *specific* for soluble antigen.

Also, in addition to the reasons given above, claim 18 should not be rejected under Farrington *et al.* because claim 18 teaches that the sporozoite is released by mechanical disruption. The method disclosed by Farrington et al. is biological disruption: "Faecal suspensions in 5 ml glass Bijou bottles were placed in a boiling water bath for 3 min, briefly allowed to settle and supernatants diluted 1:2 or 1:10 in PBS." (p. 9, last line through p. 12, first line.) The instant specification discloses that heating is considered biological disruption: "An exemplary mechanism is incubation at a temperature above room temperature." (p. 17, lines 3-7.)

The Examiner argues that "the specification does not specifically define '*specific for.*' The specification at page 9, lines 11-14 and page 10, lines 19-23 states that an antibody to *C. parvum* that is specific for viable *C. parvum* oocysts and does not react with other Cryptosporidium species or other parasites is disclosed." This phrase does not define "specific for" as only applying to Cryptosporidium species, as the Examiner states. Rather, the phrase states that the antibody is specific for viable *C. parvum* oocysts *and* does not cross-react with other species. Other places in the specification point to the meaning of the term "specific for" applying to all three elements listed in the claims, namely soluble, *C. parvum*, and sporozoites. For instance, page 7, lines 31-32, reads "The reagent is an antibody having binding specificity for a soluble *C. parvum* sporozoite antigen." Therefore, it is clear that the term "specificity for" applies to each element listed in the claim: soluble, *C. parvum*, and sporozoite. Furthermore, there is an accepted meaning of "specific" in the art. For example, the textbook Fundamental

Immunology (Paul, Raven Press, New York, 3rd Ed., Exhibit A) states that "The specificity of an antibody or antiserum is defined by its ability to discriminate between the antigen against which it was made (called the homologous antigen, or immunogen) and any other antigen one might test." (page 440, left col., 3rd par.) In the absence of a different definition of a term in the specification, the term must be interpreted in keeping with the art-accepted meaning of the term. Thus, the Examiner should give this art-accepted meaning to the term "specific for." Because the antigen for which the antibody was made is a "soluble antigen of a *C. parvum* sporozoite," only an antibody that can discriminate on all of these bases has the specificity required by the claims. . In other words, to be a properly applied 35 U.S.C. § 102 rejection, the art must teach that the antibody is specific for each of the following three elements: a soluble antigen, *C. parvum*, and a sporozoite. The Examiner has picked one element only and in so doing has not considered all of the claimed elements. For this reason, the present rejection is improper and should be withdrawn.

Furthermore, the MPEP states that "During patent examination, the pending claims must be given the broadest reasonable interpretation consistent with the specification." (MPEP 2173.05(a).) In this case, the broadest reasonable interpretation is that the antibody is *specific for* a soluble antigen of a *C. parvum* sporozoite. The Examiner cannot pick and choose which parts of the phrase "soluble antigen of a *C. parvum* sporozoite" are interpreted as being encompassed by the phrase "specific for." The claim is not limited to one element only, and by interpreting it as such, the Examiner does not give the definition the broadest reasonable interpretation

consistent with the specification, as directed by the MPEP. Therefore, applicants respectfully request reconsideration and withdrawal of this rejection.

Claims 1, 3, 4, and 17-18 are rejected under 35 U.S.C. 102(b) as allegedly being anticipated by Moss et al. The Examiner states that Moss et al. discloses rabbit antiserum against soluble proteins from *C. parvum* oocysts.

Applicants respectfully traverse. Claims 1, 3, 4, and 17-18 are drawn to a composition comprising an antibody specific for a soluble antigen of a *C. parvum* sporozoite. The antibodies of Moss et al. are not *specific* for a soluble antigen of *C. parvum* sporozoite. As discussed above, each element of the phrase encompassed by "specific for" must be taught for the rejection to be proper, and this is not the case with the Moss et al. reference. The antibodies of Moss et al. are not *specific* for soluble antigens. In fact, the abstract states the contrary: "These results show that lymphocytes from lymph nodes of mice exposed to *C. parvum* oocysts proliferate when cultured *in vitro* with soluble *or particulate antigens* prepared from oocysts." (Emphasis added). Furthermore, page 394, left column, last paragraph discloses the source of antibodies used in the experiments. Rabbit antiserum was prepared against oocysts only, not soluble portions of the oocysts. Furthermore, monoclonal antibody C8C5 disclosed on page 394, top right column, is specific for the 23-kD antigen, which is found on the surface of sporozoites, and therefore not soluble (page 393, top right column.) Also, Figure 2 shows that the antibodies of Moss et al. recognized both soluble proteins and insoluble proteins. Therefore, since both insoluble and soluble antigens were detected by the antibodies of Moss et al., the antibodies are not specific for

soluble antigen. Therefore, applicants respectfully request reconsideration and withdrawal of this rejection.

Also, in addition to the reasons given above, claim 17 should not be rejected under Moss *et al.* because claim 17 teaches that the sporozoite is released by biological disruption. The method disclosed by Moss et al. is mechanical disruption: "The oocysts were disrupted by ultrasonication...Further disruption of the sonicate was accomplished by freezing and thawing three times..." (p. 394, right col., 3rd par.) The instant specification discloses that "...oocysts are mechanically disrupted, such as by freezing and thawing..." (p. 17, lines 14-16.)

The Examiner argues that the term "specific" lacks a definition in the specification, and the claimed antibody is described as specific for viable *C. parvum* oocysts and does not cross-react with other *Cryptosporidium* species or other parasites. These arguments are addressed above.

The Examiner also states that the argument that the 23 kD antigen found on the surface of sporozoites is not soluble and thus the antibody disclosed by Moss is not specific for a soluble antigen is not persuasive. The Examiner states that nowhere in Moss is there a recitation of the 23 kD antigen not being soluble. In addition to the 23 kD antigen not being soluble, it is also not specific to sporozoites. Bonafonte et al. (Exp. Par., 96: 32-41, 2000, Exhibit B) disclose that the 23 kD antigen of *C. parvum* "is present in both the sporozoite and merozoite stages." (p. 33, left col. 2nd par.) As previously discussed, the claims state that the antibody is "specific for a soluble antigen of a *C. parvum* sporozoite." Because the 23 kD can be obtained from merozoites, it is not specific to sporozoites. An antibody that binds the 23 kD antigen is, by definition, not specific

for the sporozoite antigen. Therefore, applicants respectfully request reconsideration and withdrawal of this rejection.

Claims 1- 4, and 17-18 are rejected under 35 U.S.C. 102(b) as allegedly being anticipated by Perryman et al. The Examiner states that Perryman et al. discloses antibodies specific to *C. parvum* sporozoites.

Applicants respectfully traverse. Claims 1-4 and 17-18 are drawn to a composition comprising an antibody specific for a soluble antigen of a *C. parvum* sporozoite. The term "specific for" applies to each element listed in the claims: soluble, *C. parvum*, and sporozoite. This is the accepted meaning of "specific" in the art, as discussed above. The antibodies of Perryman et al. are not specific for a soluble antigen. On page 13, lines 8-15, Perryman et al. discloses a mAb shown to bind peptide epitopes of *C. parvum* antigens. Two distinct epitopes are found within p23, both of which are *surface glycoproteins* of *C. parvum* sporozoites. Since these epitopes are within the *surface* glycoprotein, the corresponding antibodies cannot be specific for a *soluble* antigen. Perryman et al. does not teach or suggest that the glycoprotein is soluble. Therefore, applicants respectfully request reconsideration and withdrawal of this rejection.

Also, in addition to the reasons given above, claim 17 should not be rejected under Perryman *et al.* because claim 17 teaches that the sporozoite is released by biological disruption. The method disclosed by Perryman et al. is mechanical disruption: "Genomic DNA was extracted from ... oocysts which had been previously frozen...in PBS." (p. 15, lines 14-15) The instant specification discloses that "...oocysts are mechanically disrupted, such as by freezing and thawing..." (p. 17, lines 14-16.)

The Examiner states that because the specification states that the antibody can be specific for a glycoprotein or membrane bound protein, the art of Perryman is correctly applied. However, the specification does not define "soluble" as being inclusive of glycoproteins. Instead, "glycoprotein" is one embodiment described in the specification, and not intended as a limiting definition. This embodiment was not incorporated into the claims, and therefore the claims cannot be interpreted as encompassing glycoproteins.

Furthermore, the antigen of Perryman et al. is described as p23 (p. 6, line 30 and p.13, lines 11-12). This is the same 23 kD antigen discussed above in reference to Moss et al. This antigen is not specific to sporozoites. Bonafonte et al. (Exp. Par., 96: 32-41, 2000, Exhibit B) disclose that the 23 kD antigen of *C. parvum* "is present in both the sporozoite and merozoite stages." (p. 33, left col. 2nd par.) As previously discussed, the claims state that the antibody is "specific for a soluble antigen of a *C. parvum* sporozoite." Because the 23 kD can be obtained from merozoites, it is not specific to sporozoites. Therefore, applicants respectfully request reconsideration and withdrawal of this rejection.

Claims 1- 4, and 17-18 are rejected under 35 USC 102(b) as allegedly being anticipated by Petersen et al. The Examiner states that Petersen discloses monoclonal antibodies to a soluble *C. parvum* sporozoite glycoprotein.

Applicants respectfully traverse. Claims 1-4 and 17-18 are drawn to a composition comprising an antibody specific for a soluble antigen of a *C. parvum* sporozoite. The term "specific for" applies to each element listed in the claims: soluble, *C. parvum*, and sporozoite. This is the accepted meaning of "specific" in the art, as discussed above. Petersen et al. discloses

three antibodies that react with a 900,000-M_r antigen, MAbs 10C6, 7B3, and E6 (page 5134, right col.) Petersen et al. discloses that 10C6 also reacts with intracellular merozoites. Therefore, it is not specific for a sporozoite antigen (page 5135, left col.). Petersen discloses that 7B3 also recognizes a 38,000-M_r molecule present in oocysts but not sporozoites. Therefore, it is not specific for a sporozoite antigen.

Also, in addition to the reasons given above, claim 17 should not be rejected under Petersen et al. because claim 17 teaches that the sporozoite is released by biological disruption. The method disclosed by Petersen et al. is mechanical disruption. Petersen et al. states that "Oocysts were extracted as previously described (11)." (p. 5133, right col. 1st par.) This reference is Gut et al. (J. Protozool., Vol. 38, No. 6, Nov-Dec 1991, Exhibit C). Gut et al. disclose that "For the excystation of sporozoites...oocysts were pelleted by centrifugation." (p. 72S, left col., 5th par.) Therefore, applicants respectfully request reconsideration and withdrawal of this rejection.

As discussed above, Petersen et al. discloses antibodies that are reactive with a >900,000-M *C. parvum* sporozoite glycoprotein known as gp900. However, Bonnin et al. (Parasitol. Res., 87:589-592, 2001, Exhibit D) discloses that gp900 is "an abundant glycoprotein of *C. parvum* merozoites and sporozoites." (Abstract.) Therefore, the antibodies directed against gp900 would not have been specific for a *C. parvum* sporozoite, as they would have also been directed against merozoites. Therefore, applicants respectfully request reconsideration and withdrawal of this rejection.

Claims 1- 4 and 17-18 are rejected under 35 U.S.C. 102(b) as allegedly being anticipated by Riggs et al. The Examiner states that Riggs et al. discloses compositions comprising monoclonal antibodies to *C. parvum* sporozoites.

Applicants respectfully traverse. Claims 1-4 and 17-18 are drawn to a composition comprising an antibody specific for a soluble antigen of a *C. parvum* sporozoite. Riggs et al. discloses five MAbs were found to give a posteriorly capped staining on sporozoites with posteriorly extruded fluorescent material, suggesting the presence of shed antigens. However, Riggs et al. also discloses that "these [five] MAbs were found to bind to oocyst walls." (page 7, lines 20-24). Therefore, these monoclonal antibodies are not specific for sporozoites as they also bind oocyst walls. The term "specific for" applies to each element listed in the claims: soluble, *C. parvum*, and sporozoite. This is the accepted meaning of "specific" in the art, as discussed above. Therefore, applicants respectfully request reconsideration and withdrawal of this rejection.

Also, in addition to the reasons given above, claims 17 and 18 should not be rejected under Riggs *et al.* because claim 17 teaches that the sporozoite is released by biological disruption, and claim 18 teaches that the sporozoite is released by mechanical disruption. Riggs *et al.* does not teach how the sporozoite is released, but merely states that the *C. parvum* sporozoites had been solubilized in lysis buffer (p. 5, line 22). This does not indicate the means of release from the oocyst. Since no method or release was taught, the rejection is inappropriately applied to these claims. Therefore, applicants respectfully request reconsideration and withdrawal of this rejection.

The Examiner states that the fact that some of the antibodies disclosed by Riggs have cross reactivity with oocyst walls is not relevant since the claims do not exclude such a possibility. However, the claims do exclude such a possibility, as they recite "an antibody specific for a soluble antigen of a *C. parvum* sporozoite." As discussed above, each element recited must be present in the art for an anticipatory rejection to be appropriate. In other words, to be a properly applied 35 U.S.C. § 102 rejection, the art must teach that the antibody is specific for a soluble antigen, specific for *C. parvum*, and specific for a sporozoite. The art of Riggs et al. does not teach that the antibody is specific for a sporozoite, as the antibody of Riggs cross-reacts with oocyst walls. Therefore, applicants respectfully request reconsideration and withdrawal of this rejection.

Claims 1- 4 and 17-18 are rejected under 35 USC 102(b) as allegedly being anticipated by Tilley et al. The Examiner states that Tilley discloses monoclonal antibodies that bind sporozoite surface and apical complex antigens.

Applicants respectfully traverse. Claims 1-4 and 17-18 are drawn to a composition comprising an antibody specific for a soluble antigen of a *C. parvum* sporozoite. Tilley et al. teaches antibodies against sporozoite surface, apical surface, and inner oocyst wall (Table 1). Those antibodies reactive against sporozoite and apical surface are not soluble by definition, as they are present on the surface of the bacteria. Furthermore, these antibodies were shown to react with both the sporozoite and merozoite surface (page 238, left col.) and are therefore not sporozoite specific. The one antibody against the internal oocyst wall, mAb2D7, was not only "unstable" but also not shown to be specific (page 238, right col.).

Also, in addition to the reasons given above, claims 17 and 18 should not be rejected under Tilley *et al.* because claim 17 teaches that the sporozoite is released by biological disruption, and claim 18 teaches that the sporozoite is released by mechanical disruption. Tilley *et al.* does not teach that the sporozoite is released at all: "Supernatants from wells displaying growth were tested for anti-C. parvum antibodies...against air dried sporozoites and excysted and non-excysted oocysts." (p. 236, left col, 1st par.) Since no method or release was taught, the rejection is inappropriately applied to these claims. Therefore, applicants respectfully request reconsideration and withdrawal of this rejection.

The Examiner states that the claims are not limited to antibodies that do not have any cross-reactivity. However, the claims recite "an antibody *specific for* a soluble antigen of a *C. parvum* sporozoite," and therefore do exclude any cross-reactivity. As discussed above, each element recited must be present in the art for an anticipatory rejection to be appropriate. In other words, to be a properly applied 35 U.S.C. § 102 rejection, the art must teach that the antibody is specific for a soluble antigen, specific for *C. parvum*, and specific for a sporozoite. The art of Tilley et al. does not teach that the antibody is specific for a sporozoite, as the antibody of Tilley et al. cross-reacts with oocyst walls. Therefore, applicants respectfully request reconsideration and withdrawal of this rejection.

Pursuant to the above remarks, reconsideration and allowance of the pending application is believed to be warranted. The Examiner is invited and encouraged to directly contact the undersigned if such contact may enhance the efficient prosecution of the application to issue.

A Credit Card Payment Form PTO-2038 authorizing payment in the amount of $790.00, representing the filing fee is enclosed. This amount is believed to be correct; however, the commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 14-0629.

Respectfully submitted,

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Michael Laird — 12/13/04 (Date)

Exhibit A

antigen 1. Excess antibody is washed off, and enzyme-labeled antigen 2 is added. If bound antibody cross-reacts with both antigen 1 and antigen 2, then enzyme-labeled antigen 2 will adsorb onto the solid phase. This will be measured as increased absorbance of the enzyme-catalyzed reaction. This method has been used to assay the cross-reactivity of anti-idiotype antisera for two preparations of idiotype, or for detection of anti-anti-idiotype (see Chapters 9 and 24). Other arrangements of antibody and antigen are also possible. Extra layers of detecting reagents can amplify sensitivity but also tend to raise the background and introduce variability.

An example of the first method described above is the detection of human antibodies to influenza virus (52) (Fig. 10). Alternate columns were coated with influenza virus or bovine albumin. Serum was added at 1/10 dilution to the top two wells of each box and serially diluted in fourfold steps from top to bottom. The last colored well indicates the titer, whereas the absence of color in the albumin-coated wells indicates the specificity. A second use of this method is for screening culture supernatants in the production of hybridoma antibodies. The sensitivity and speed of the ELISA method make it possible to screen large numbers of wells for the production of specific antibody. Clones selected by this method tend to have high antigen affinities, perhaps due to dissociation of low-affinity antibodies during the wash steps.

An important caution when using native protein antigens to coat solid-phase surfaces (Fig. 9A) is that binding to a surface can alter the conformation of the protein. For instance, using conformation specific monoclonal antibodies to myoglobin, Darst et al. (53) found that binding of myoglobin to a surface altered the apparent affinity of some antibodies more than others. This problem may be avoided by using the methods of Fig. 9C.

SPECIFICITY AND CROSS-REACTIVITY

The specificity of an antibody or antiserum is defined by its ability to discriminate between the antigen against which it was made (called the homologous antigen, or immunogen) and any other antigen one might test. In practice, one cannot test the whole universe of antigens, but only selected antigens. In this sense, specificity can only be defined experimentally within that set of antigens one chooses to compare. Karush (28) has defined a related term, selectivity, as the ability of an antibody to discriminate, in an all-or-none fashion, between two related ligands. Thus selectivity depends not only on the relative affinity of the antibody for the two ligands but on the experimental lower limit for detection of reactivity. For instance, an anti-carbohydrate antibody with an affinity of $10^5\ M^{-1}$ for the immunogen may appear to be highly selective, since reaction with a related carbohydrate with a 100-fold lower affinity, $10^3\ M^{-1}$, may be undetectable. On the other hand, an antibody with an affinity of $10^9\ M^{-1}$ for the homologous ligand may appear to be less selective because any reaction with a related ligand with a 100-fold lower affinity would still be quite easily detectable.

Conversely, cross-reactivity is defined as the ability to react with related ligands other than the immunogen. More usually, this is examined from the point of view of the ligand. Thus one might say that antigen Y cross-reacts with antigen X because it binds to anti-X antibodies. Note that in this sense, it is the two antigens that are cross-reactive, not the antibody. However, the cross-reactivity of two antigens, X and Y, can be defined only with respect to a particular antibody or antiserum. For instance, a different group of anti-X antibodies may not react at all with Y, so that with respect to these antibodies, Y would not be cross-reactive with X. One can also use the term in a different sense, saying that some anti-X antibodies cross-react with antigen Y.

In most cases, cross-reactive ligands have lower affinity than the immunogen for a particular antibody. However, exceptions can occur, in which a cross-reactive antigen binds with a higher affinity than the homologous antigen itself. This phenomenon is called heteroclicity, and the antigen that has a higher affinity for the antibody than does the immunogen is said to be heteroclitic. Antibodies that manifest this behavior are also described as heteroclitic antibodies. A good example is the case of antibodies raised in C57BL/10 mice against the hapten nitrophenyl acetyl (NP). These antibodies have been shown by Mäkelä and Karjalainen (54) to bind with higher affinity to the cross-reactive hapten, nitroiodophenyl acetyl (NIP), than to the immunogen itself.

In many practical situations, cross-reactivity is detected by methods such as precipitin, especially precipitation in agar (the Ouchterlony test), or hemagglutination (see below for descriptions of both of these) or similar methods, which have in common the fact that they do not distinguish well between differences in affinity and differences in concentration. This practical aspect, coupled with the heterogeneity of immune antisera, has led to ambiguities in the usage of the terms "cross-reactivity" and "specificity." With the advent of RIA techniques, this ambiguity in the terminology, as well as in the interpretation of data, has become apparent.

For these reasons, Berzkofsky and Schechter (55) have defined two forms of cross-reactivity and, correspondingly, two forms of specificity. These two forms of cross-reactivity are illustrated by the two prototype competition RIA curves in Fig. 11. In reality, most antisera display both phenomena simultaneously.

Type 1 cross-reactivity, or true cross-reactivity, is defined as the ability of two ligands to react with the same site on the same antibody molecule, possibly with different affinities. For example, the related haptens dinitrophenyl (DNP) and trinitrophenyl (TNP) may react with

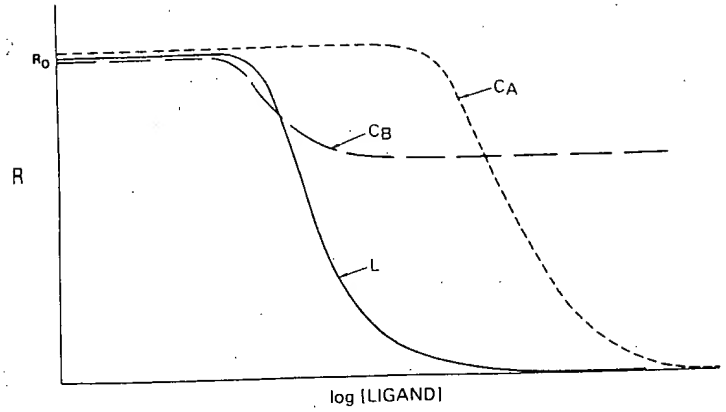

FIG. 11. Schematic RIA binding curves for homologous ligand L and cross-reacting ligands. Cross-reacting ligand C_A manifests type 1 or true cross-reactivity demonstrated by complete inhibition of tracer ligand binding, and a lower affinity. Ligand C_B displays type 2 cross-reactivity or determinant sharing, as recognized from the plateau at less than 100% inhibition, but not necessarily a lower affinity. The ordinate R is the ratio of bound/free radiolabeled tracer ligand, and R_0 is the limit of R as the concentration of all ligands, including tracer, approaches zero. (From ref. 55, with permission.)

different affinity for antibodies raised to dinitrophenyl hapten. In protein antigens, such differences could occur with small changes in primary sequence (e.g., the conservative substitution of threonine for serin), or with changes in conformation, such as the cleavage of the protein into fragments (Fig. 12). If a peptide fragment contained all the contact residues in an antigenic determinant (i.e., those which contact the antibody-combining site), it might cross-react with the native determinant for antibodies against the native form, but with lower affinity because the peptide would not retain the native conformation (see Chapter 8). This type of affinity difference is illustrated by competitor C_A in Fig. 11, in which complete displacement of tracer can be achieved at high enough concentrations of C_A, but higher concentrations of C_A than of the homologous ligand, L, are required to produce any given degree of inhibition.

A separate issue from affinity differences is the issue of whether the cross-reactive ligand reacts with all or only a subpopulation of the antibodies in a heterogeneous serum. This second type of cross-reactivity, which we call type 2 cross-reactivity or shared reactivity, therefore can occur only when the antibody population is heterogeneous, as in most conventional antisera. In this case, the affinity of the cross-reactive ligand may be greater than, less than, or equal to that of the homologous ligand for those antibodies with which it interacts. Therefore the competition curve is not necessarily displaced to the right, but the inhibition will reach a plateau at less than complete inhibition, as illustrated by competitor C_B in Fig. 11. As an example, let us consider the case of a protein with determinants X and Y, and an antiserum against this protein containing both anti-X and anti-Y antibodies. Then a mutant protein in which determinant Y was so altered as to be unrecognizable by anti-Y, but determinant X was intact, would manifest type 2 cross-reactivity. It would compete with the wild-type protein only for anti-X antibodies (possibly even with equal affinity), but not for anti-Y antibodies.

Occasionally, even monoclonal antibodies may appear to display type 2 cross-reactivity in situations in which secondary reactions are involved in the measurement of the antigen–antibody reaction. For example, Sharon et al. (60) and Cisar et al. (61) observed plateau values at less than 100% binding of a homogeneous myeloma or hybridoma antibody reactive with dextrans. In this case, the assay used was quantitative precipitin, in which differential solubility of different complexes could account for such a plateau. If one could directly observe the antigen–antibody interaction in solution, without the need for any secondary reaction that might be incomplete, the reaction of a homogeneous antibody with its homogeneous antigen theoretically cannot reach a plateau at less than 100% reaction or inhibition. Therefore the existence of secondary competing reactions should be considered when such plateaus are observed.

Of course, both types of cross-reactivity could occur simultaneously. A classic example would be the peptide fragment discussed in the case of type I cross-reactivity above. Suppose the fragment contained the residues of determinant X, albeit not in the native conformation, but did not contain the residues of a second determinant, Y, which was also expressed on the native protein. If the antiserum to the native protein consisted of anti-X and anti-Y, the peptide would compete only for anti-X antibodies (type 2 cross-reactivity) but would have a lower affinity than the native protein even for these antibodies. Thus the competition curve would be shifted to the right and would plateau before reaching complete inhibition.[8]

In the case of a homogeneous (e.g., monoclonal) antibody in which only type 1 or true cross-reactivity can occur, one can quantitate the differences in affinity for different cross-reactive ligands by a method analogous to

[8] An ambiguous case could occur experimentally in which the distinction between the two types of cross-reactivity would be blurred. For example, in the case of antibodies that all react with determinant X but have a very wide range of affinities for X, some such antibodies may have such a low affinity for cross-reactive determinant X′ that they would appear not to bind X′ at all. Then a competition curve using X′ might appear to reach a plateau at incomplete inhibition even though all the antibodies were specific for X, and the only difference between X and X′ was affinity.

the B/F versus F method described above. Suppose that ligands X and Y cross-react with homologous ligand L for a monoclonal antibody. If one plots the bound/free (B/F = R) ratio for radiolabeled tracer ligand L as a function of the log of the concentration of competitors X and Y, one obtains two parallel competition curves (Fig. 13), under the appropriate conditions (below). The first condition is that the concentration of free tracer be less than $1/K_L$, the affinity for tracer. In this case, it can be shown (55) that

$$K_X \simeq \frac{1}{[X]_{free}} \qquad [41]$$

at the midpoint where $R = R_0/2$, where K_X is the affinity for X. This is analogous to Equation (21) for the case in which unlabeled homologous ligand is the competitor. Also, in analogy with Equation (23), it can be shown that if the total concentration of competitor, $[X]_t$, is used instead of the free concentration, $[X]_{free}$, an error term will arise, giving

$$[X]_t \text{ (at } R = R_0/2) = \frac{1}{K_X} + \frac{[S]_t}{2} \qquad [42]$$

Thus, with competitor on a linear scale, the difference in midpoint for competitors X and Y will correspond to the difference $1/K_X - 1/K_Y$ regardless of whether free or total competitor is plotted, but the ratio of midpoint concentrations will equal K_X/K_Y only if the free concentrations are used. This last point is important if one plots the log of competitor concentration, as is usually done, since the horizontal displacement between the two curves on a log scale corresponds to the ratio $[X]/[Y]$, not the difference (55).

If a second condition also holds, namely, that the concentration of bound tracer is small compared to the antibody site concentration $[S]_t$, then the slopes (on a linear scale) of the curves at their respective midpoints (where $R = R_0/2$) will be proportional to the affinity for that competitor, K_X or K_Y (55). (Both conditions can be met by keeping tracer L small relative to both K_L and $[S]_t$.) When $[X]_{free}$ and $[Y]_{free}$ are plotted on a log scale, the slopes will appear to be equal (i.e., the curves will appear parallel), since a parallel line shifted m-fold to the right on a log scale will actually be $1/m$ as steep, at any point, in terms of the antilog as abscissa.

When the antibodies are heterogeneous in affinity, the curves will be broadened and in general will not be parallel. When heterogeneity of specificity is present, and type 2 cross-reactivity occurs, it should be pointed out that the fractional inhibition achieved at the plateau in a B/F versus free competitor plot will not be proportional to the fraction of antibodies reacting with that competitor but will be proportional to a weighted fraction, where the antibody concentrations are weighted by their affinity for the tracer (55).

These two types of cross-reactivity lead naturally to two definitions of specificity (55). The overall specificity of a heterogeneous antiserum is a composite of both of these facets of specificity. Type 1 specificity is based on the relative affinities of the antibody for the homologous ligand and any cross-reactive ligands. If the affinity is

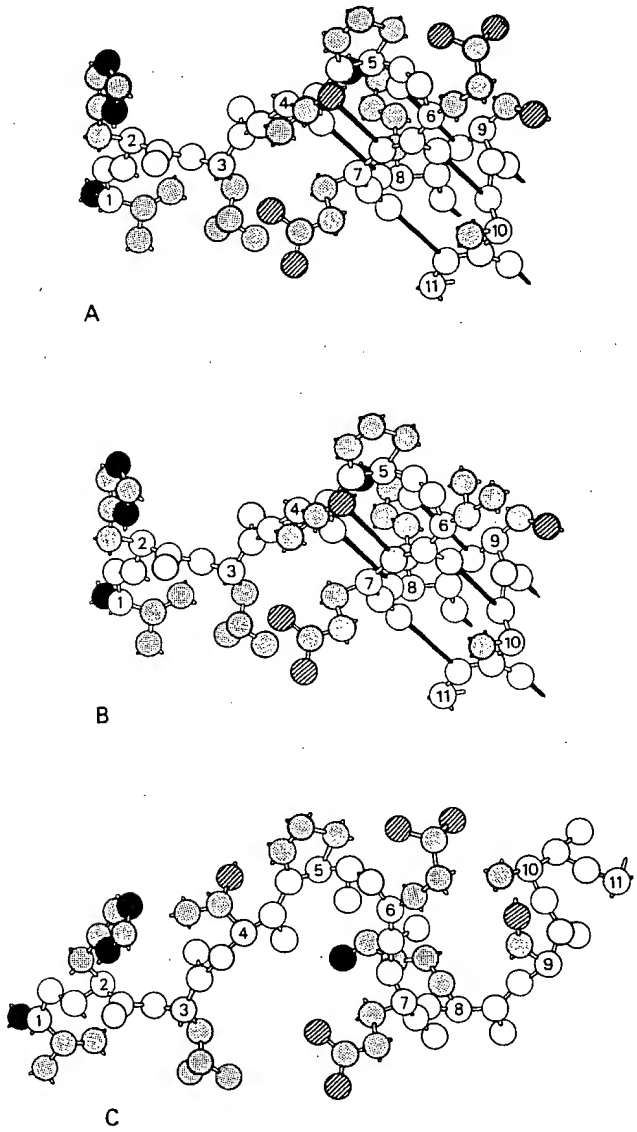

FIG. 12. An artist's drawing of the amino terminal region of the β chain of hemoglobin. **A:** The first 11 residues of the β^A chain. **B:** The comparable regions of the β^S chain. The substitution of valine for the normal glutamic acid at position 6 makes a distinct antigenic determinant to which a subpopulation of antibodies may be isolated (56,57). **C:** A schematic diagram of the sequence in (A) unfolded as occurs when the protein is denatured. This region may be cleaved from the protein, or the peptide synthesized (58), resulting in changed antigenic reactivity. An antiserum prepared to hemoglobin (or the β chain thereof) might exhibit cross-reactivity with the structures shown in (B) and (C) but the molecular mechanisms would be different. Polypeptide backbone atoms are in *white;* in the side chains, oxygen atoms are *hatched,* nitrogen atoms are *black,* and carbon atoms are *lightly stippled.* (Adapted from refs. 55 and 59.)

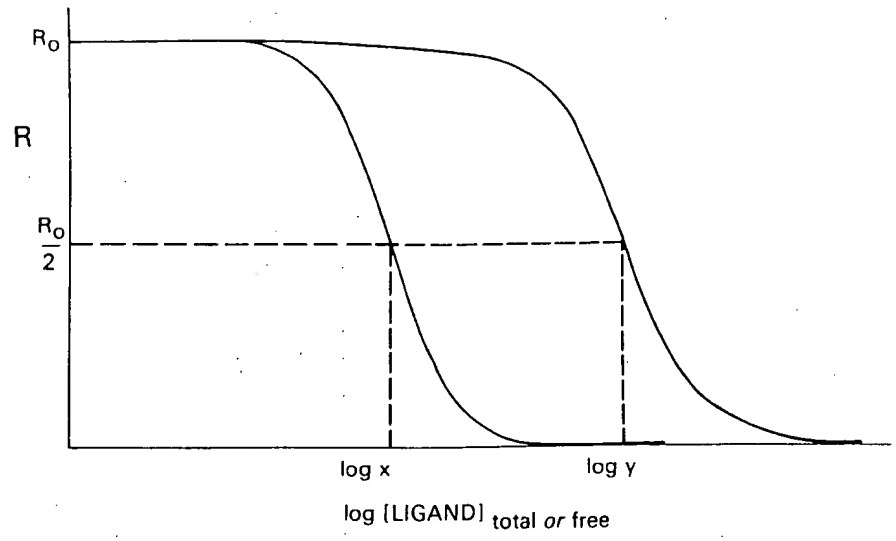

FIG. 13. Schematic RIA binding curves showing the effect of affinity on the midpoint and the slope at the midpoint, and the value of using free [ligand] rather than total [ligand]. Ordinate R is the ratio of bound/free radiolabeled tracer ligand, and R_0 the limit of R as all ligand concentrations approach zero. If x and y are the concentrations of ligands X and Y that reduce R to exactly $R_0/2$, then if the abscissa is total ligand concentration, $x = 1/K_X + [S]_t/2$ and $y = 1/K_Y + [S]_t/2$, where $[S]_t$ is the concentration of antibody binding sites and K_X and K_Y the affinities of the antibody for the respective ligands. However, if the abscissa is free ligand concentration, $x = 1/K_X$ and $y = 1/K_Y$ so that the ratio x/y (or the difference log x − log y on a log plot) corresponds to the ratio of affinities K_Y/K_X. Note that the slopes at the midpoints are the same on a log scale, but that for Y would be only K_Y/K_X that for X on a linear scale. (From ref. 55, with permission.)

much higher for the homologous ligand than for any cross-reactive ligand tested, then the antibody is said to be highly specific for the homologous ligand; that is, it discriminates very well between this ligand and the others. If the affinity for cross-reactive ligands is below the threshold for detection in an experimental situation, then type 1 specificity gives rise to selectivity as was discussed above (cf. ref. 28). The specificity can even be quantitated in terms of the ratio of affinities for the homologous ligand and a cross-reactive ligand (cf. ref. 62). It is this type 1 specificity that most immunochemists would call true specificity, just as we have called type 1 cross-reactivity true cross-reactivity.

The common use of the term "cross-reactivity" to include type 2 or partial reactivity leads to a second definition of specificity, which applies only to heterogeneous populations of antibodies such as antisera. We call this type 2 specificity. If all the antibodies in the mixture react with the immunogen, but only a small proportion react with any single cross-reactive antigen, then the antiserum would be said to be relatively specific for the immunogen. Note that it does not matter whether the affinity of a subpopulation that reacts with a cross-reactive antigen is high or low (type 1 cross-reactivity). As long as that subpopulation is a small fraction of the antibodies, the mixture is specific. Thus type 2 specificity depends on the relative concentrations of antibodies in the heterogeneous antiserum, not just on their affinities. Also note that one can use these relative concentrations of antibody subpopulations to compare the specificity of a single antiserum for two cross-reactive ligands. However, it would not be meaningful to compare the specificity of two different antisera for the same ligand by comparing the fraction of antibodies in each serum which reacted with that ligand. Although type 2 specificity may appear to some a less classic concept of specificity than type 1, it is type 2 specificity that one primarily measures in such assays as the Ouchterlony double immunodiffusion test, and it carries equal weight with type 1 specificity in such assays as hemagglutination, discussed below. Type 2 specificity also leads naturally to the concept of "multispecificity" described below.

Multispecificity

The theory of multispecificity, introduced and analyzed by Talmadge (63) and Inman (64,65) and discussed on a structural level by Richards et al. (66), suggests a mechanism by which the great diversity and specificity of antisera can be explained without the need for a correspondingly large repertoire of antibody structures (or structural genes). The idea is that each antibody may actually bind, with high affinity, a wide variety of quite diverse antigens. When one immunizes with immunogen A, one selects for many distinct antibodies,

which have in common only that they all react with A. In fact, each antibody may react with other compounds, but if fewer than 1% of the antibodies bind B, and fewer than 1% bind C, and so on, then by type 2 specificity, the whole antiserum will appear to be highly specific for A. Note that the subpopulation that binds B may react with an affinity for B as high as or higher than that for A, so that the population would not be type 1 specific for A. This same population would presumably be selected if one immunized with B, as well as with perhaps hundreds of other immunogens with which these antibodies react. The net result would be that the diversity of highly (type 2) specific antisera an organism could generate would be much greater than the diversity of B cell clones (or antibody structures) that it would require. However, this concept of multispecificity remains an interesting hypothesis without experimental validation (see ref. 55 and references therein).

OTHER METHODS

We mention only a few of the other methods for measuring antigen–antibody interactions. Other useful techniques include quenching of the tryptophan fluorescence of the antibody by certain antigens on binding (67) (a sensitive method useful for such experiments as fast kinetic studies), antibody-dependent cellular cytotoxicity, immunofluorescence including flow cytometry, immunohistochemistry, and inhibition by antibody of plaque formation by antigen-conjugated bacteriophage (68) (a method as sensitive as RIA since inhibition of even a few phage virions can be detected).

Quantitative Precipitin

Among the earliest known properties of antibodies were their ability to neutralize pathogenic bacteria and their ability to form precipitates with bacterial culture supernatants. Both activities of the antiserum were highly specific for the bacterial strain against which the antiserum was made. The precipitates contained antibody protein and bacterial products. The supernatants contained decreased amounts of antibody protein and, under the right conditions, had lost the ability to neutralize bacteria. However, quantitation of the antibody precipitated was difficult, since the precipitate contained antigen protein as well as antibody protein. Heidelberger and Kendall (69,70) solved this problem when they found that purified pneumococcal cell wall polysaccharide could precipitate with anti-pneumococcal antibodies. In this case, the amount of protein nitrogen measured in the precipitate was entirely due to antibody nitrogen. Plotting the amount of antibody protein precipitated from a constant volume of antiserum by increasing amounts of carbohydrate antigen gives the curve shown in Fig. 14.

As shown in Fig. 14A, the amount of antibody precipitated rises initially, reaches a plateau, then falls off. The point of maximum precipitation was found to coincide with the point of complete depletion of neutralizing antibodies and is called the equivalence point. The amount of antibody protein in the precipitate at equivalence is considered to equal the total amount of specific antibody in that volume of antiserum. The rising part of the curve is called the antibody excess zone (antigen limiting), and the part of the curve beyond the equivalence point is called the antigen excess zone.

Careful analysis of supernatants and precipitates was carried out for each zone of antibody or antigen excess, as shown in Fig. 14B. When antigen was limiting, the precipitate contained high ratios of antibody to antigen. The supernatant in this zone contained free antibody with no detectable antigen. As more antigen was added, the amount of antibody in the precipitate rose, but the ratio of antibody to antigen fell. At equivalence, no free

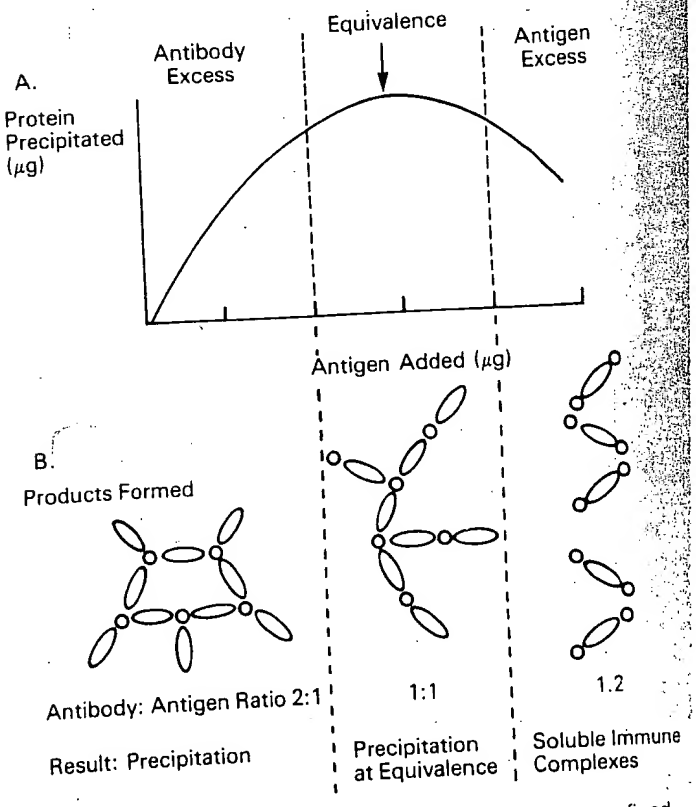

FIG. 14. Quantitative immunoprecipitation. To a fixed amount of specific antibody are added increasing amounts of nonprotein antigen. The figure shows the amount of antibody protein (**A**) and the ratio of antibody to antigen (**B**) found in the precipitate. At antigen excess, soluble immune complexes are found in the supernatant, and the precipitate is decreased.

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A 23-kDa Recombinant Antigen of *Cryptosporidium parvum* Induces a Cellular Immune Response on *in Vitro* Stimulated Spleen and Mesenteric Lymph Node Cells from Infected Mice

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Department of Pediatrics, Emory University School of Medicine, Atlanta, Georgia 30033, U.S.A.; and Veterans Affairs Medical Center, Decatur, Georgia 30033, U.S.A.

Bonafonte, M.-T., Smith, L. M., and Mead, J. R. 2000. A 23-kDa recombinant antigen of *Cryptosporidium parvum* induces a cellular immune response on *in vitro* stimulated spleen and mesenteric lymph node cells from infected mice. *Experimental Parasitology* **96**, 32–41. In the present study, we focused on a 23-kDa antigen, Cp23, which has been shown to be a major target of humoral immune responses in *Cryptosporidium parvum* infections and is present in both the sporozoite and merozoite stages. Recombinant Cp23 antigen was shown to stimulate a specific proliferative response by splenocytes and mesenteric lymph node cells from infected interferon gamma knockout BALB/c mice. Cp23 stimulation also induced TNF-α, IL-2, and IL-5 mRNA production by spleen cells from infected animals. In contrast, IL-12 mRNA was decreased by Cp23 stimulation compared with unstimulated splenocytes. These data suggest that, as with humoral responses, Cp23 is an important target of cellular immune responses in experimental *C. parvum* infections. The potential role of this antigen in conferring protective immunity is also discussed.

Index Descriptors and Abbreviations: C. parvum, Cryptosporidium parvum; apicomplexa; recombinant antigen; cellular immune response; Cp23, *C. parvum* 23-kDa recombinant antigen; MLN, mesenteric lymph node cells; IFN-γ, interferon gamma; GKO, knockout; IL-, interleukin; TNF-α, tumor necrosis factor alpha; PCR, polymerase chain reaction; DNA, deoxyribonucleic acid; GST, glutathione S-transferase; *E. coli, Escherichia coli*; FBS, fetal bovine serum; PBS, phosphate-buffered saline; BCA, bicinchoninic acid assay; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel; HRP, horseradish peroxidase; HEPA, high-efficiency particulate-air; pi, postinfection; BSA, bovine serum albumin; ConA, concanavalin A; SAg, sporozoite antigen; ELISA, enzyme-linked immunosorbent assay; HPRT, hypoxanthine phosphoribosyltransferase; RT–PCR, reverse transcriptase PCR; cDNA, complementary deoxyribonucleic acid; mRNA, messenger RNA; ssDNA, single strand DNA; cpm, counts per minute.

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INTRODUCTION

Cryptosporidium parvum is an opportunistic protozoan that infects the gastrointestinal tract of humans and other mammals (Fayer and Ungar, 1986). Although the life cycle of *C. parvum* takes place in the enterocytes of the intestinal epithelium, the immune response to this parasite can also be analyzed at the level of spleen cells (Iochmann *et al.*, 1999; Sagodira *et al.*, 1999; Tilley *et al.*, 1995; Harp *et al.*, 1994; Chen *et al.*, 1993), mesenteric lymph node cells (Davami *et al.*, 1997), and intraepithelial lymphocytes (Chai *et al.*, 1999; McDonald *et al.*, 1996).

The spectrum of illness is directly linked to the immune status of the host. The infection is self-limiting in immunocompetent hosts but is often severe and protracted in immunocompromised hosts (Fayer and Ungar, 1986; O'Donoghue, 1995). Despite intensive screening of potential therapeutics, no specific treatments or preventive measures are currently available; this is in part due to the limited knowledge of the mechanisms of immunity to this parasite. Although both humoral and cellular responses may play a role in host recovery from infection, it is apparent that neither the intensity nor the duration of infection is dependent on

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the magnitude of the antibody response (Taghi-Kilani *et al.*, 1990), suggesting that the role of antibodies in resolution of infection is minor. In contrast, persistent cryptosporidiosis infection in mammals with impaired cellular immune response capability provides indirect evidence that T cells are essential for recovery from infection (Current and Garcia, 1991). For example, studies with athymic mice provided evidence for the importance of T lymphocytes in recovery from cryptosporidial infection (Ungar *et al.*, 1991, 1990). Additional experiments have shown that $CD4^+$ T lymphocytes are involved in recovery from infection (Harp *et al.*, 1994; Mead *et al.*, 1991; Ungar *et al.*, 1991). Similarly, T cells are critical for protective immunity against related coccidia (*Eimeria and Toxoplasma*) (Rose *et al.*, 1991; Suzuki *et al.*, 1990), both as helper cells to stimulate immunoglobulin production and as effector cells through the release of soluble mediators.

To date, the study of immune responses to specific antigens of *C. parvum* has been restricted to the characterization of serologic reactivity. Humoral responses to antigens described by several groups recognize a number of immunodominant sporozoite antigens, including polypeptides of approximately 11, 15, 23, 44, 100, 180, and > 200 kDa (Mead and You, 1998; Peeters *et al.*, 1992; Hill, 1990). There is, however, little knowledge regarding the nature of specific antigens that induce protective responses (Perryman *et al.*, 1999; Sagodira *et al.*, 1999) during natural infection or of the precise cellular responses that mediate protective immunity. Data concerning the identity of *C. parvum* recombinant antigens which stimulate T lymphocytes remain scarce (Iochmann *et al.*, 1999; Gomez-Morales *et al.*, 1995).

In the present study, we focused on Cp23, a major target of humoral immunity. This antigen is present in both the sporozoite and merozoite stages (Arrowood *et al.*, 1991, 1989) and is considered a marker of infection since it is recognized by serum antibodies of humans and many animal species (Priest *et al.*, 1999; Riggs *et al.*, 1994; Mead *et al.*, 1998, 1988; Lumb *et al.*, 1988). More recently, antibodies directed against Cp23 were also shown to demonstrate protection (Enriquez and Riggs, 1998). Using cells from infected mice bearing a targeted disruption of the IFNγ-gene, we evaluated proliferative and cytokine responses to recombinant Cp23. Unlike adult immunocompetent mice that are refractible to infection and suckling mice that recover quickly from infection, mice bearing a targeted disruption of the IFN-γ gene are remarkably susceptible to infection with *C. parvum* (Smith *et al.*, 2000; Mead and You, 1998; Theodos *et al.*, 1997). Even though these mice are immunodeficient, they recover from infection in a 3- to 4-week period (Mead and You, 1998). The immune response in reconstituted mice was also evaluated to assess the effect of IFN-γ in proliferation. IFN-γ-GKO-BALB/c mice were reconstituted with spleen cells from naïve BALB/c mice and the proliferative response in Cp23-stimulated spleen cells and MLN was compared with that of infected non-reconstituted and noninfected reconstituted IFN-γ-GKO-BALB/c.

One limitation of this study is that IFN-γ may have an important role in host defense of cryptosporidiosis but the lack of IFN-γ did not affect the capacity to eliminate *C. parvum* infection.

MATERIALS AND METHODS

Recombinant Cp23 expression and purification. Clone Cp23 was kindly provided by Drs. Jeff Priest and Patrick Lammie, Centers for Disease Control and Prevention, Atlanta, Georgia. Cp23 was PCR amplified from *C. parvum* genomic DNA with primers 5′-ATG-GGT-TGT-TCA-TCA-TCA-3′ (sense primer) and 5′-TCG-GTC-GAC-TAC-GGA-TT-3′ (antisense primer) designed from GenBank sequence U34390 and cloned in the expression vector pGEX-4T (Amersham Pharmacia Biotech, Piscataway, NJ) in frame with *Schistosoma japonicum* glutathione S-transferase using standard techniques. Expression of the protein was accomplished by first inoculating 10 ml of 2xYT (1.6% tryptone, 0.5% NaCl, and 1.0% yeast) supplemented with 2% glucose and 100 μg/ml ampicillin with *Escherichia coli* containing pGEX-4T/Cp23 or control pGEX-4T. Cultures were incubated overnight at 37°C while shaking. Ten milliliters of the overnight culture was then added to 1 liter of supplemented 2xYT media and incubated at 37°C until the appropriate density was reached (A_{600} = 0.5). Protein expression was induced with 0.1 mM isopropyl-β-D-thiogalactopyranoside, and the culture incubated for 2 h at 37°C while shaking. The culture was centrifuged at 7700*g* for 10 min, and the bacterial pellet was resuspended in 30 ml of ice-cold PBS and sonicated on ice until clear. The sonicate was centrifuged at 12,000*g* at 4°C for 10 min and the supernatant was saved for purification. The recombinant protein and the control GST were purified with a bulk GST purification module (Amersham Pharmacia Biotech) according to the manufacturer's instructions. Thrombin protease was added to the concentrated samples to cleave GST. The *C. parvum* Cp23 protein was released by overnight cleavage with thrombin at room temperature and separated from uncleaved fusion protein and the GST cleavage product by passage over glutathione Sepharose 4B resin. The protein concentration of purified Cp23 was determined using a BCA assay (Pierce, Rockford, IL).

Western blot. The purified Cp23 (5 μg) and GST proteins were separated on a 10% SDS–PAGE gel and electroblotted to a nitrocellulose membrane to assess protein purity. Two identical blots were prepared and both were blocked with 5% milk buffer. One blot was incubated with rabbit anti-*Cryptosporidium* polyclonal antibody (1:200) and the other was incubated with goat anti-GST (1:1000) (Amersham Pharmacia Biotech). The appropriate secondary antibodies conjugated to biotin were added at a 1:1000 dilution. The proteins were detected by incubation with streptavidin conjugated to HRP (1:1000) with tetramethylbenzidine. Biotinylated molecular weight markers (Bio-Rad, Hercules, CA) were detected with avidin–HRP.

Animals. Six- to eight-week-old male IFN-γ-GKO (BALB/c-*Ifg*^{tm1}) mice (Jackson Laboratories, Bar Harbor, ME) were used. The GKO mice were fed sterile food and water and kept in HEPA-filtered barrier-isolated facilities. Manipulations for these mice were performed in HEPA-filtered, biological containment hoods. Knockout mice were kept in microisolator cages and acclimated for 1 week prior to oocyst inoculation. All mice were housed at the Veterans Affairs Medical Center (Decatur, GA) animal facility.

Parasite and inoculation. The *C. parvum* isolate used for this study was the Iowa bovine isolate. Oocysts were collected and purified through discontinuous sucrose and cesium chloride gradients as previously described (Arrowood and Donaldson, 1996). Purified oocysts were stored at 4°C in a 2.5% potassium dichromate ($K_2Cr_2O_7$) aqueous solution. Oocyst inocula were prepared by washing purified oocysts (stored < 6 months) with 0.1% BSA, PBS (pH 7.2) to remove potassium dichromate.

BALB/c-*Ifg*^{tm1} were inoculated with 2×10^6 oocysts by oral gavage. For reconstitution experiments, GKO mice were injected with 10^7 splenocytes from naïve BALB/c mice 2 days after infection with *C. parvum*. Fecal samples were collected from the mice at 3-day intervals and assessed for parasite load by flow cytometry as previously described (Arrowood *et al.*, 1995). BALB/c-*Ifg*^{tm1} mice were sacrificed on days 14 and 28 postinfection.

For proliferation assays and *in vitro* splenocyte stimulation for cytokine production, oocysts were washed 3× in 0.1 M acetate/NaCl buffer and centrifuged at 14,000 rpm for 3 min. The oocyst pellet was suspended in 100 μl fresh (10×) $NaIO_4$ and 900 μl 0.1 M acetate/NaCl buffer. This was incubated on ice for 20 min, and then 0.1% BSA/PBS was added and the mixture centrifuged for 3 min. The pellet was resuspended in 0.1 M acetate/NaCl buffer, 75 μl of NaT (0.75% final) was added, and this was incubated at 37°C for 10 min. The excysted sporozoites were separated from the unexcysted oocysts and empty shells by passage through a sterile 3.0-μM-pore-size polycarbonate filter (Poretics Corp., Livermore, CA). The purified sporozoites were washed 2× with PBS and sonicated with three bursts (10 s) at 50% duty. The debris was removed by centrifugation and the concentration of sporozoite antigen in the supernatant was determined by BCA (Pierce).

Lymphocyte proliferation. Proliferative responses of spleen cells from BALB/c-*Ifg*^{tm1} to antigen (Con A, positive control; Cp23; and SAg) were measured by the uptake of [^{3}H]thymidine 4 days after stimulation with the aforementioned antigens. Spleens were harvested from individual control (n = 5) and infected (n = 5) or infected reconstituted (n = 5) animals and dissociated into a single cell suspension using beveled glass slides. The spleen cells (2×10^5 cells per well) from mice were placed in 96-well plates (Costar, Cambridge, MA) in complete RPMI 1640 containing 10% heat-inactivated fetal bovine serum (Mediatech, Herndon, VA), 200 mM L-glutamine, 100 U penicillin/streptomycin (Mediatech), and 5×10^{-5} M 2-mercaptoethanol. Antigens were added at the following final concentrations: 0.1, 1, and 10 μg/ml. The total volume per well was 200 μl. The plates were kept in a humidified CO_2 incubator (5% CO_2, 95% air) at 37°C for 4 days. Eighteen hours before harvest of the cultures, the cells were pulsed with 1 μCi of [^{3}H]thymidine. The cells were harvested onto glass fiber filter paper with a multiple automated sample harvestor (Packard, Meriden, CT) and incorporation of [^{3}H]thymidine was assessed with a Matrix 9600 beta counter (Packard). Proliferative responses of MLN from INF-γ-GKO-BALB/c to Cp23 antigen and SAg were measured as with spleen cells. MLN cells were harvested from individual noninfected control (n = 10), infected (n = 10), and infected reconstituted (n = 5) mice. Antigens were evaluated at 1 or 10 μg/ml.

ELISA IFN-γ measurement. Concentrations of IFN-γ from supernatant fluid of *in vitro* stimulated splenocytes were measured by OptEIA ELISA kit (Pharmingen, San Diego, CA). Briefly, 96-well ELISA plates (Nunc, Maxisorp, Roskilde, Denmark) were coated overnight at 4°C with anti-IFN-γ capture antibody according to the manufacturer's instructions. Nonspecific binding was decreased by incubating the plates with 10% FBS in PBS (200 μl/well) for 1 h. Supernatant fluids from stimulated splenocytes were collected and 50 μl was added to each well. Each sample was performed in triplicate. The supernatant fluids and IFN-γ standards were incubated for 2 h at room temperature. The plates were washed extensively before the addition of biotinylated anti-mouse IFN-γ detection antibody. The antibody was detected with avidin–horseradish peroxidase conjugate with tetramethylbenzidine (Kirkegaard and Perry Laboratories, Gaithersburg, MD) as the substrate. The reaction was stopped after 30 min with 2 N H_2SO_4 and the assay was read on a Bio-Kinetics Plate Reader (Bio-Tech Instruments, Winooski, VT) at 450 nm with λ correction at 570 nm.

Isolation and purification of total RNA. Duplicate pools of splenocytes (1×10^7 cells/24-well) from infected (n = 5) and noninfected (n = 5) mice were stimulated *in vitro* with ConA (2.5 μg/ml), Cp23 (2.5μg/ml), SAg (2.5 μg/ml), or medium alone for 48 h. Initial experiments were performed to determine a suitable concentration range of antigen to be used in lymphocyte proliferation studies. Total RNA was isolated as recommended by the manufacturer (TriReagent, Molecular Research Center, Inc., Cincinnati, OH). The RNA was resuspended in diethylpyrocarbonate-treated water and quantitated spectrophotometrically.

Reverse transcriptase–PCR detection of cytokine mRNA. A reverse transcriptase–PCR (RT–PCR) was performed to determine relative quantities of mRNA for the cytokines interleukin-2 (IL-2), IL-4, IL-5, and IL-12, tumor necrosis factor alpha (TNF-α), IFN-γ, and the housekeeping gene hypoxanthine phosphoribosyltransferase (HPRT). Reverse transcription of RNA was performed in a 25-μl final volume. Reaction tubes contained (1) 2.5 μl, 2.5 mM mix of all four deoxynucleotides (Boehringer Mannheim, Indianapolis, IN); (2) 5 μl 5× reverse transcriptase buffer, 50 mM Tris–HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$ (Gibco, BRL, Gaithersburg, MD); (3) 2 μl, 0.1 M DTT (Gibco, BRL); (4) 0.5 μl RNAsin (Promega, Madison, WI); (5) 2 μl random hexamer (Boehringer Mannheim); (6) 1 μl Superscript reverse transcriptase (Gibco, BRL). RNA and H_2O in a final volume of 12 μl were heated at 70°C for 5 min and chilled on ice for 5 min. RNA samples were added to the reaction tubes, incubated at 37°C for 60 min, heated to 90°C for 5 min to denature the reverse transcriptase, cooled on ice for 5 min, and stored at −20°C. The final reaction volume was diluted 1:8 by the addition of 175 μl of distilled H_2O.

The primers and probes for all genes have been described (Bost and Clements, 1995; Wynn *et al.*, 1993) and were prepared using a DNA synthesizer at Emory University Microchemical Facility (Atlanta, GA). To the PCR reaction mixture the following components were added: (1) 0.25 mM dNTP mix; (2) 1× PCR buffer (50 mM KCl, 10 mM Tris–HCl, 1.5 mM $MgCl_2$; (3) 30 pmol sense and antisense primers; (4) 10 μl cDNA; (5) 1 unit *Taq* polymerase (Promega). After an initial incubation at 95°C for 3 min, temperature cycling was initiated as follows: (1) 94°C for 1 min; (2) specific temperature for each cytokine for 1 min; (3) 72°C for 2 min. Those steps were repeated 30 to 40 times depending on cytokine. PCR reaction conditions were defined for each cytokine. For each gene product, the optimum number of cycles was determined experimentally and was defined as that number of cycles that would achieve a detectable concentration that was well below saturating conditions. The housekeeping gene HPRT was reverse

transcribed and amplified in each assay to verify that equal amounts of cDNA were added in each PCR reaction. The number of PCR cycles selected for each cytokine gene were as follows: IL-2 (35), IL-4 (33), IL-5 (32), IL-12 (40), TNF-α (32), IFN-γ (30), and HPRT (30).

After the amplification PCR products were analyzed by Southern blotting, a total of 13.2 μl of the final reaction mix was run on a 1% agarose gel at 70 V for 1.5 h. The gel was then denatured by soaking for 30 min in 1.5 M NaCl, 0.5 N NaOH with gentle agitation. The gel was rinse in deionized water and neutralized by soaking for 30 min in 1 M Tris, pH 7.4, 1.5 M NaCl at room temperature with gentle agitation. The DNA was transferred to a nylon membrane (Amersham, Arlington Heights, IL) using an electrotransfer system (USA Scientific, Ocala, FL). The membrane was UV cross-linked using the UV Stratalinker 1800 (Stratagene, La Jolla, CA) and baked at 80°C for 2 h in an oven. Blots were prehybridized at 42°C for 6 h in a solution containing: 6× SSPE, 10× Denhardt's, 1% SDS, and 50 g/ml ssDNA. Blots were hybridized with ^{32}P-labeled oligoprobes at 49°C for 16 h in a solution containing 6× SSPE and 1% SDS. Probes were selected to hybridize to a portion of the amplified segment between the primers. After hybridization, blots were washed for 15 min in 6× SSPE, 0.1% SDS and then 4 min in 2× SSPE at 49°C. Autoradiography signal was detected using an Instantimager (Packard). The resulting bands represent areas of specific probe hybridization. The band intensity in cpm was quantitated by direct nuclear counting and results were reported as percentages of cytokine mRNA relative to HPRT.

Statistics. The differences in the proliferative responses of control, infected, and reconstituted mice were analyzed with Student's *t* test.

RESULTS

Expression of Cp23 and Western blot reactivity with rabbit sera. After expression, Cp23 and GST control were purified and analyzed by SDS–PAGE and Western blot. Two identical blots were prepared; one was incubated with rabbit anti-*Cryptosporidium* polyclonal antibody (Fig. 1) and the second blot was incubated with goat anti-GST (specific reactivity to the purified 27-kDa GST protein was observed, data not shown). Similar to Priest *et al.* (1999), who evaluated serologic specimens using the 23-kDa recombinant antigen, Fig. 1 demonstrates specific reactivity of anti-*Cryptosporidium* polyclonal antibodies with proteins of ~ 23 kDa (cleaved protein) and of ~46 kDa (purified fusion protein, GST/Cp23 after partial thrombin cleavage). The Cp23 sequence which codes for an 11.2-kDa protein (Perryman *et al.*, 1996) has been shown on SDS–gels and Western blots to migrate as a 27-kDa (Priest, *et al.*, 1999; Perryman *et al.*, 1996) or 23-kDa (Perryman *et al.*, 1996; Lumb *et al.*, 1988; Mead *et al.*, 1988) protein, depending on the gel conditions. No reactivity of the *C. parvum*-specific serum was detected in the lane loaded with GST control (Fig. 1, lane 5).

Proliferative response. Differences in responsiveness of spleen cells to Cp23 were evaluated at 2 and 4 weeks pi.

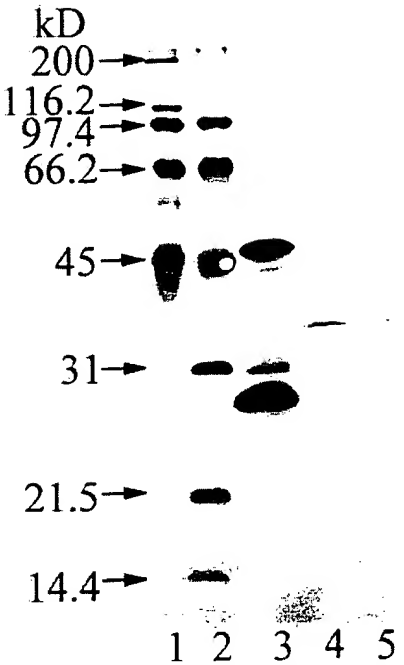

FIG. 1. The purified Cp23 was separated on a 10% SDS–PAGE gel and electroblotted to a nitrocellulose membrane. The blot was incubated with rabbit anti-*Cryptosporidium* polyclonal antibody. Lanes: 1, high molecular weight; 2, low molecular weight; 3, 5 μg of Cp23, anti-*Cryptosporidium parvum* polyclonal antibody reacted with proteins of ~23 and ~46 kDa; 4, negative control (media) for reactivity; 5, GST control, no reactivity of anti-*C. parvum* antibody was noted with the GST control. Biotinylated molecular weight markers (10^3) are depicted on the left.

Phenotypic analysis by flow cytometry of splenocytes from control and infected mice demonstrated no consistent differences in cell populations between infected and control mice at either 2 or 4 weeks pi (data not shown). Proliferation of splenocytes from *C. parvum*-infected mice demonstrated a fourfold increase ($P \leq 0.001$) above controls in response to Cp23 (Fig. 2A). Figure 2B shows the proliferative response of splenocytes from infected reconstituted, infected, and control mice at 2 weeks pi to Cp23. There was a fivefold increase ($P \leq 0.05$) above controls in spleen cells from infected reconstituted mice and a threefold increase ($P > 0.05$) in spleen cells from infected non-reconstituted mice (Fig. 2B). When the proliferative responses of spleen cells from infected reconstituted and infected non-reconstituted mice to Cp23 (Fig. 3A) and to SAg (Fig. 3B) were compared to one another, the difference was not statistically significant.

The response observed to the 23-kDa antigen in the control wells was not significant, demonstrating that the presence

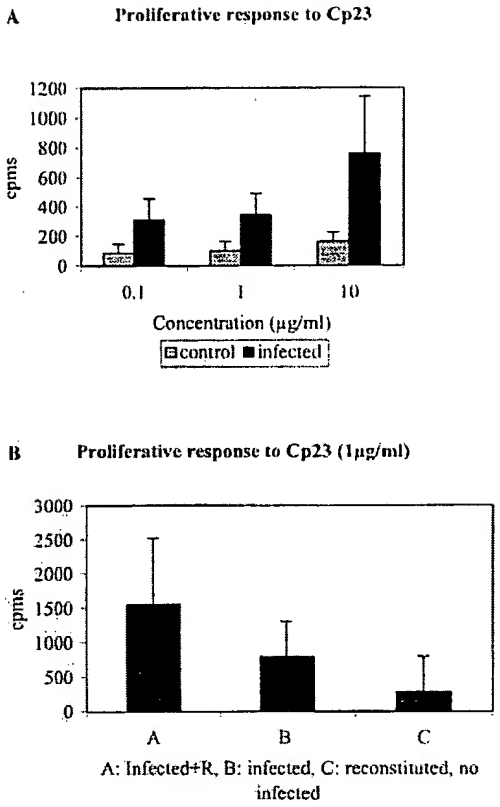

FIG. 2. Splenocytes from IFN-γ-GKO-BALBc mice infected (2 weeks) with *C. parvum* or sham infected were restimulated *in vitro* with Cp23 (A). (B) The proliferative response of splenocytes from control reconstituted, infected, and infected reconstituted mice at 2 weeks pi to Cp23. Proliferation was assessed by the addition of [^{3}H]thymidine at 96 h after restimulation. Results are presented as the mean thymidine incorporation (cpm) of triplicate wells stimulated with Cp23 − mean cpm of media control ± the standard deviation (SD). Each animal (n = 5) was investigated separately and the mean determined.

of uncleaved GST or any bacterial contaminant in the recombinant antigen preparation generated a minimal nonspecific reactivity. The proliferative response to Cp23 of infected animal splenocytes was still observed at 4 weeks pi but was greatly diminished to a less than twofold increase of infected over control wells (data not shown).

Responses to Cp23 and SAg were also evaluated in MLN at 2 weeks pi (Figs. 3 and 4). There was a sixfold increase ($P \leq 0.001$) between infected cells and noninfected controls when stimulated with Cp23 (Fig. 4); this was similar to the proliferation obtained in spleen cells (Fig. 2B). Differences in the proliferative response to Cp23 and SAg, between MLN cells from infected and infected reconstituted animals (Figs. 3C and 3D), were not statistically significant.

Production of IFN-γ by reconstituted GKO splenocytes: ELISA IFN-γ measurement. Spleen cells from reconstituted and non-reconstituted mice were isolated and incubated *in vitro* with ConA, for 48 h, to examine IFN-γ production in tissue culture supernatant fluids. Splenocytes from *C. parvum*-infected reconstituted mice produced 1506 pg/ml, whereas splenocytes from noninfected reconstituted mice produced 66 pg/ml. IFN-γ was not detected in splenocyte supernatants from reconstituted mice after *in vitro* stimulation with Cp23, probably due to the sensitivity of the ELISA test. As expected, IFN-γ was not detected in splenocyte supernatants from non-reconstituted mice after *in vitro* stimulation with ConA. IFN-γ expression was also determined by RT–PCR (Fig. 5). Spleen cells from infected reconstituted mice showed prominent INF-γ mRNA expression when stimulated *in vitro* with Cp23 or ConA (Fig. 5). INF-γ mRNA was not detected in infected non-reconstituted mice but was detected at low levels in the control reconstituted noninfected mice (data not shown).

Cytokine expression. Figure 6 shows the expression of cytokine mRNA in splenocytes from animals at 2 week pi after 48 h *in vitro* stimulation with Cp23 (2.5 μg/ml), the nonspecific mitogen ConA (2.5 μg/ml), or medium alone. Detectable levels of cytokine mRNA in unstimulated spleen cells were observed. Several of these cytokine mRNA levels (IL-2, IL-12, and TNF-α) were elevated in the unstimulated spleen cells from infected mice compared to control animals (Figs. 6A, 6D, and 6E). This increase was most apparent (over a threefold increase) with IL-12 (Fig. 6D). Cp23 stimulation of splenocyte cultures induced increases in cytokine mRNA expression. In particular, there was a sixfold increase in IL-5 mRNA expression from Cp23-stimulated splenocytes from infected animals compared with the noninfected control (Fig. 6C). IL-2 and TNF-α mRNA expression were also increased in the splenocytes from infected animals compared with the controls (panels A and E) but to a much lesser degree. Similar patterns of expression were observed with native SAg stimulation (Smith *et al.*, 2000). In contrast to unstimulated splenocyte cultures, increases in expression of IL-12 mRNA were not observed in the Cp23-stimulated splenocytes (panel D). Moreover, Cp23 stimulation appeared to have some inhibitory effect on IL-12 mRNA expression and since mRNA levels were consistently below those of unstimulated splenocytes, this was a specific immune response. Cells were not immunosuppressed since stimulation with ConA resulted in increased cytokine mRNA expression for IL-4, IL-5, and TNF-α in infected animals.

Similar to the decrease observed in proliferative responses at 4 weeks pi, there was a marked decrease in the mRNA expression of all cytokines at 4 weeks after infection. These

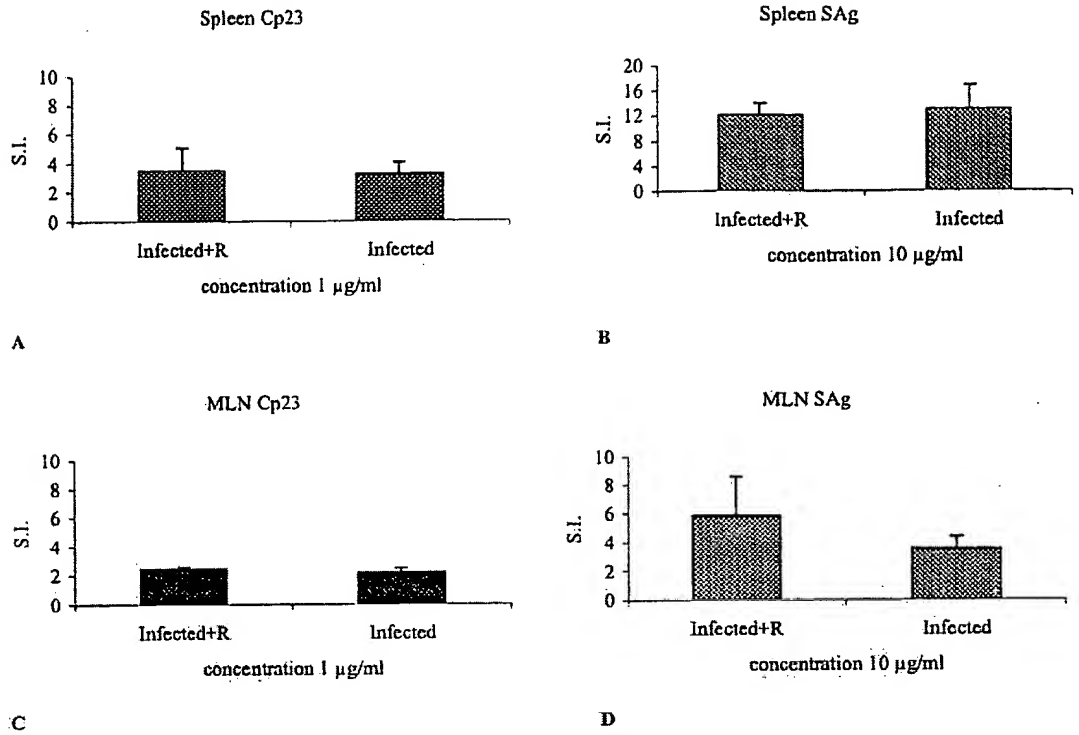

FIG. 3. Proliferative response of IFN-γ-GKO-BALBc mice to Cp23 and SAg 2 weeks pi. Splenocytes or MLN were restimulated *in vitro* with Cp23 (A and C) or SAg (B and D); proliferative response from infected reconstituted (Infected+R) and infected mice was assessed by the addition of [^{3}H]thymidine at 96 h after restimulation. Each animal (n = 5) was investigated separately and the mean of triplicate wells was determined. The results were expressed as the stimulation index, which is the ratio of proliferation of restimulated lymphocytes to the proliferation of the media alone ± the standard deviation (SD).

cytokines were weakly visible by blot, generating a signal approximately 1/4 the amount of that observed at 2 weeks pi for most cytokines (data not shown).

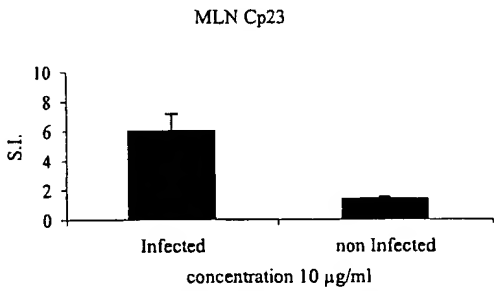

FIG. 4. MLN from mice infected (n = 10) with *C. parvum* or sham infected (n = 10) were restimulated *in vitro* with Cp23. Proliferation was assessed by the addition of [^{3}H]thymidine at 96 h after restimulation. Proliferative response of MLN is represented as the mean from 10 mice (2 weeks pi). The results were expressed as the stimulation index, which is the ratio of proliferation of restimulated lymphocytes to the proliferation of the media alone ± the standard deviation (SD).

DISCUSSION

Most of the published *ex vivo* analyses of *Cryptosporidium* splenic T cell responses have used *Cryptosporidium* sporozoite or oocyst extracts (Smith *et al.*, 2000; Tatalick and Perryman, 1995; Harp *et al.*, 1994). Until recently, few *Cryptosporidium* parasite recombinant proteins have been studied

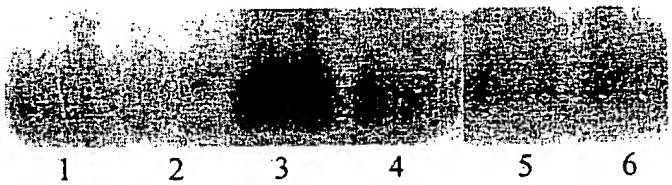

FIG. 5. IFN-γ expression (Southern blot) at 2 weeks pi in splenocytes from IFN-γ-GKO-BALB/c mice infected with *C. parvum* and reconstituted. Splenocyte were *in vitro* stimulated with ConA or Cp23. Lanes: 1, 2, unstimulated; 3, 4, ConA; 5, 6, Cp23.

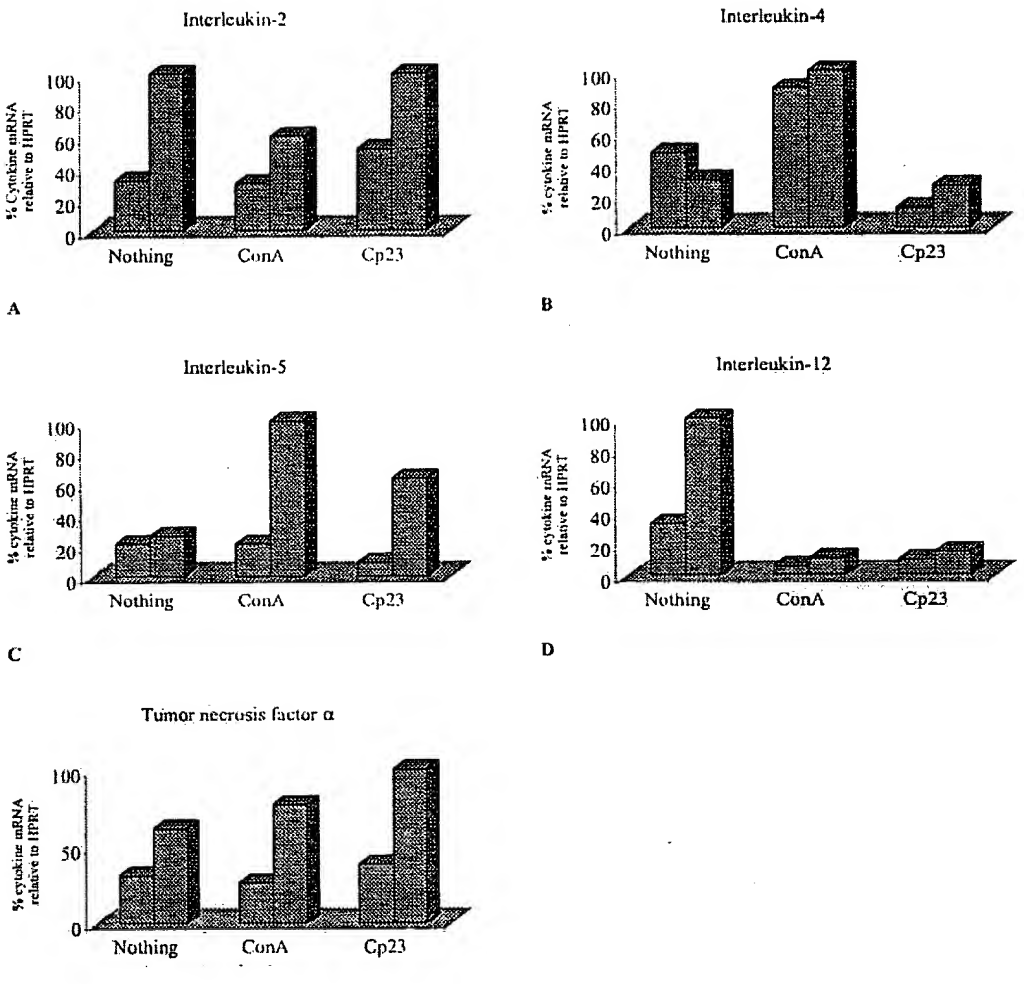

FIG. 6. Relative intensity of cytokine mRNA in the spleen of uninfected mice (control, non-reconstituted) and mice infected (non-reconstituted) with *C. parvum*. Splenocytes from a pool of five mice were *in vitro* stimulated with media alone (nothing), ConA, or Cp23 for 48 h. The relative intensity of the amplified products is expressed as the percentage after comparison to the relative intensity of the internal control housekeeping gene, HPRT. These values (average cpm) were obtained from Southern blots (data not shown) and analyzed by the Instantimager.

for their ability to induce proliferation and cytokine expression of spleen and MLN cells (Iochmann *et al.*, 1999). Gomez-Morales *et al.* (1995) described a 190-kDa recombinant antigen from *Cryptosporidium* oocysts that induced proliferation of human peripheral blood mononuclear cells *in vitro*.

The identification and characterization of *C. parvum* proteins should provide additional information about the immune response against this parasite. In this study, Cp23, an antigen localized to the surface of sporozoites and merozoites (Arrowood *et al.*, 1991, 1989) which stimulates neutralizing antibody responses (Theodos *et al.*, 1997; O'Donoghue, 1995; Enriquez and Sterling, 1993), was evaluated for its ability to induce cellular immune responses. The gene encoding Cp23, previously isolated (Perryman *et al.*, 1996) and cloned (Priest *et al.*, 1999), was expressed in *E. coli*. The fusion protein reacted with anti-*Cryptosporidium* polyclonal rabbit sera, showing that the antigenic properties were still intact. The Cp23 antigen was shown to induce a specific proliferative response in splenocytes and MLN from infected and reconstituted IFN-γ-GKO-BALB/c mice. *In vitro* blastogenic responses to *C. parvum* have been detected in spleen and lymph nodes from infected mice (Theodos *et*

al., 1997; Davami *et al.*, 1997; Tilley *et al.*, 1995) and in peripheral blood lymphocytes from infected calves (de Graff *et al.*, 1998; Whitmire *et al.*, 1991). Sagodira *et al.* (1999) obtained a potent *C. parvum*-specific blastogenesis in stimulated spleen T cells from mice immunized with a 15-kDa (CP15) recombinant antigen. They also obtained a similar response with cells from MLN; however, the proliferative response of splenocytes was greater than that of MLN. Our results show a similar proliferative response from both spleen and MLN cells. These experiments suggest that the spleen, a more distant organ from the site of infection, may be as relevant as MLN for studying certain cellular immune responses. This is further corroborated by Sagodira *et al.* (1999), who found CP15-DNA-immunized mice developed specific and long-lasting anti-CP15 IgA and mice also developed an antigen-specific T lymphocyte proliferative response in both spleen and MLN.

We demonstrated that splenocytes from infected mice proliferated in response to Cp23 in an antigen-specific and dose-dependent manner. The increase in specific proliferation of spleen cells and MLN in infected mice to Cp23 antigen correlated with the increased of cytokine mRNA expression. At 4 weeks pi as parasite load decreased, lymphocyte proliferation and cytokine expression also decreased. There was a small decrease in the proliferation and cytokine mRNA expression by spleen cells from infected reconstituted mice when compared to spleen cells from infected mice; parasite load was also decreased. There was not a significant difference in the proliferation of stimulated spleen cells from infected reconstituted and infected mice, but these small differences may be due to the time point analyzed.

The susceptibility of mice to disease appears to be affected by the genetically controlled differentiation of Th cells into Th1 or Th2 cells (Mosmann and Coffman, 1989). Few studies of cryptosporidiosis have examined Th2 cytokines (Aguirre *et al.*, 1998; Huang *et al.*, 1996; Enriquez and Sterling, 1993). Our analyses of cytokine mRNA revealed different patterns of expression in infected and uninfected (control) mice. There was a modest increase in IL-2 expression over controls and a more marked increase in IL-5 in splenocytes stimulated with Cp23. In contrast to our observations, Tilley *et al.* (1995) did not detect any cytokines in supernatants from control spleen cells and no splenic IL-4 production was detected by ELISA in mice repeatedly challenged with oocysts of *C. parvum* (Urban, *et al.*, 1996). These differences may be explained by the different sensitivities of the assays used in each study or by the different mouse model. In this study, an IFN-γ-GKO-BALB/c mouse model (Smith *et al.*, 2000; Mead and You, 1998) was used, which excludes IFN-γ-dependent mechanisms of immune response to infection. IFN-γ may have an important role in host defense against cryptosporidiosis; however, since these mice were able to recover, IFN-γ-independent mechanisms (Th2 cytokines) may also be involved in recovery. A study by Ungar *et al.* (1991) using a BALB/c mouse model treated with anti-IFN-γ concluded that while IFN-γ was important in recovery from infection, clearance occurred via IFN-γ-independent effector mechanisms as well.

Detectable levels of cytokine mRNA in unstimulated spleen cells were observed. This increase was most apparent (over threefold increase) with IL-12. Gazzinelli *et al.* (1994) detected high levels of spontaneous cytokine secretion by peritoneal and spleen cells harvested from mice at 5 days and cultured *in vitro* in the absence of *Toxoplasma gondii* antigen. They also detected low levels of cytokine mRNAs in both peritoneal and spleen cells from mice before infection (naïve). Preliminary studies with IL-10 cytokine show that IL-10 expression in infected and infected reconstituted cells stimulated with Cp23 was similar to IL-10 expression in cells stimulated with SAg. When stimulated with Cp23, IL-10 expression in infected and infected reconstituted cells was higher than when stimulated with ConA or when cells were not stimulated (data not shown).

Previous studies demonstrated that *C. parvum* sporozoite extracts reacted with Cp23-specific mAbs. While *C. parvum* responses to native proteins have been described in humans, mice, cattle, and other mammals, relatively few studies have examined responses to *C. parvum* recombinant antigens (Gomez-Morales, *et al.*, 1995; Iochmann, *et al.*, 1999). Sporozoite extracts, containing the native Cp23 protein, and recombinant Cp23 may differ antigenically and contain different epitopes that are recognized by T cells, but our results show that Cp23 induces a cellular immune response similar to that of native SAg (Smith *et al.*, 2000).

The capacity of an antigen to mediate protection depends on qualitative and quantitative differences in host responses. The protective antigen may generate a different profile of cytokines (Th1 or Th2) or may stimulate the release of cytokine levels necessary for elimination of the organism. One study showed (Allendoerfer *et al.*, 1996) that mice immunized with a yeast protein reacted *in vivo* to the antigen but cells from these animals did not recognize the antigen *in vitro*. Various recombinant proteins confer protective immunity when used as vaccines (Gomez *et al.*, 1995; Sagodira *et al.*, 1999) and this may be possible with the Cp23 protein (Perryman *et al.*, 1999). Perryman *et al.* (1999) demonstrated that immunization with a recombinant protein of *C. parvum* containing the amino acid sequence of the major part of Cp23 induced immune bovine colostrum that protected calves against cryptosporidiosis following oral challenge

with *C. parvum* oocysts. However, further studies are needed to determine if immunization with the recombinant protein protects mice against infection. This study was performed in INF-γ-GKO-BALB/c mice and the results are pertinent exclusively to murine cryptosporidiosis. In conclusion, Cp23 induces humoral and cellular responses and may be an important antigen in eliciting protection from *C. parvum* infection.

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Exhibit C

Cryptosporidium parvum: In Vitro Cultivation in Madin-Darby Canine Kidney Cells

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SUMMARY. To facilitate studies of the biology of *Cryptosporidium parvum*, we have developed an in vitro culture system using Madin-Darby canine kidney (MDCK) cells as the host cell. Oocysts or free sporozoites were incubated at 37° C with monolayers of MDCK cells in supplemented RPMI 1640 medium and the cells were examined at various time intervals after initiation of the culture. High rates of infection (up to 90% of MDCK cells) were achievable. Sequential development of trophozoites, meronts, microgametocytes, and macrogametocytes was observed over a 72-h period of culture. Between 72 and 96 h we observed formation of oocyst walls, but fully sporulated oocysts were not observed. This culture system provides access to both the asexual and sexual intracellular stages of *C. parvum*.

Key words. Trophozoites, meronts, microgametocytes, macrogametocytes.

CRYPTOSPORIDIUM parvum (Cp) is an intracellular coccidian parasite that infects intestinal epithelial cells and causes a clinical syndrome of diarrhea. A single species infects humans and a variety of other mammals [2]. In immunocompetent hosts, infection is usually self-limited and diarrhea is transient. In immunocompromised hosts, especially those with AIDS, infection may persist and cause life-threatening dehydration and malabsorption. There is currently no effective treatment for cryptosporidiosis.

Cryptosporidium parvum infection is initiated by sporozoites that excyst from oocysts in the stomach and duodenum and invade the microvillous border of intestinal epithelial cells. Invasion is followed by intracellular development of the parasite within a parasitophorous vacuole and includes asexual multiplication and sexual differentiation. Asexual multiplication produces meronts containing merozoites. Merozoites rupture into the intestinal lumen and reinvade intestinal epithelial cells. Gamogony occurs when some merozoites develop into microgametocytes (males) and macrogametocytes (females) rather than undergoing merogony. Fertilization of macrogametocytes results in new oocyst production. Most oocysts are shed in the feces and are capable of transmitting the infection to new hosts. Some oocysts may rupture in the intestinal lumen, resulting in a cycle of autoinfection [2].

Research on *C. parvum* has been conducted primarily with oocysts isolated from the feces of infected animals or with intracellular stages from the small intestine of experimentally infected animals. However, studies of the biology and treatment of cryptosporidiosis would be facilitated by an in vitro culture system supporting sporozoite invasion and intracellular development within an appropriate host cell. Here we report an in vitro culture system with Madin-Darby canine kidney (MDCK) cells that yields large numbers of asexual and sexual stages of *C. parvum*.

MATERIALS AND METHODS

Our studies were performed predominantly with oocysts isolated from calves infected with the AUCP-1 strain of *C. parvum*, which was a generous gift from Dr. Byron Blagburn, Auburn University, Auburn, Alabama. Oocysts were purified by filtration and density gradient centrifugation with sucrose, sterilized by incubation with undiluted commercial bleach for five min at 4° C., washed x 5 with RPMI 1640 medium (GIBCO, Grand Island, NY), and stored in RPMI 1640 at 4° C until used.

For the excystation of sporozoites, 10^7 oocysts were pelleted by centrifugation (1000 *g* for 10 min, 4° C) and resuspended with 0.75% sodium taurocholate in RPMI-1640, pH 6.0, in a 15-ml conical centrifuge tube. The tube was gassed with 3% O_2, 6% CO_2, 91% N_2 and incubated 20 min at 37° C. Sodium bicarbonate was added to raise the pH to 7.3 and the tube was again incubated for 20 min at 37° C. Excystation was monitored by interference-contrast microscopy. The excystation of oocysts varied between 10% and 90%, depending on the sample. To isolate sporozoites, the excystation mixture was filtered through a 3 µm polycarbonate filter that traps oocysts. Sporozoites from the filtrate were pelleted by centrifugation (1000 *g*, 10 min, room temperature), resuspended in Cp culture media (vide infra), counted in a hemocytometer, and aliquoted for culture.

Cell lines used in these experiments were from the American Type Culture Collection (ATCC) or the University of California, San Francisco, Cell Culture Facility. The cells were cultured in the recommended media or RPMI-1640 supplemented with 10% bovine serum (Hyclone), penicillin G (100 U/ml), streptomycin (100 µg/ml), L-glutamine (2 mM), sodium pyruvate (0.11 mg/ml), and buffered with sodium bicarbonate (2.5 g/l).

For studies of infection and intracellular development, cells were harvested by trypsinization, resuspended in media, counted, and aliquoted at 10^5 cells per 1-cm² chamber of four-chamber glass microscope slides. Cells were permitted to adhere for 2 - 4 h and the media was replaced with Cp culture medium. The Cp culture media was RPMI 1640 supplemented as described above (but with less serum). In addition, Cp culture media also contained 25 mM HEPES, non-essential amino acids, insulin (0.24 U/ml), 13.6 mg/l of hypoxanthine, 2.9 mg/l of thymidine, 2-mercaptoethanol (50 µM), yeast extract (0.1%), Ficoll 400 (1%), glucose (4.5 g/l), bovine calf serum (1%), and rabbit bile (0.1%). Cells were cultured until they reached confluency (24 - 48 h) prior to addition of *C. parvum*.

Cultures were initiated by adding excysting oocysts or sporozoites in Cp culture media to confluent monolayers of cells. Following a two-hour incubation, the monolayers were washed with Cp culture media to remove non-invasive sporozoites and residual oocysts and incubated at 37° C in 6% CO_2 in air for various times. The slides were washed with phosphate-buffered saline (PBS) and examined as wet mounts by interference-contrast microscopy or by bright field microscopy after fixation with 3.5% formaldehyde in PBS (pH 7.4) for 5 min and Giemsa staining.

RESULTS

To evaluate the ability of different cell lines to support the development of *C. parvum*, we added the parasites to monolayers of 15 continuous cell lines and examined Giemsa-stained cells 24 h later. Infection was indicated by the identification of any intracellular parasite (uninucleate trophozoite, multinucleate meront, or meront containing mature merozoites). Of the 15 cell lines tested, 11 were infected, but only a small proportion of host cells were infected with most of the lines (less than 0.1%). In contrast, two kidney cell lines (Madin-Darby canine kidney and Madin-Darby bovine kidney) were heavily infected, with up to 90% of MDCK cells containing intracellular stages.

To investigate the development of intracellular stages of *C. parvum*, we inoculated monolayers of MDCK cells as described above and

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examined the cells at various times. Meronts with 1, 2 or 4 nuclei were observed between 1 - 6 h and meronts with 8 nuclei were first observed at 6 - 10 h. Fully developed meronts with 8 merozoites were first observed at 10 - 14 h and increased in numbers up to 48 h. Subsequently, we observed a predominance of sexual stages. Microgametocytes with 16 nuclei were first observed at 42 h and rapidly developed until there were 16 elongated microgametes. Macrogametocytes and macrogametes also appeared at 42 h and increased in numbers until, by 72 h, over 90% of intracellular parasites were macrogametocytes or macrogametes. Between 72 and 96 h, we observed the formation of oocyst walls, but fully sporulated oocysts were not observed. All developmental stages were found in culture for at least 7 days , but in decreasing numbers.

DISCUSSION

In vitro development of *C. parvum* was previously described with human fetal lung cells (Flow 2000) [1], mouse L929 fibroblasts [4], and with a cloned line of intestinal epithelial cells derived from a human colon carcinoma (HT29.74 cells) [3]. We report here that *C. parvum* infects monolayers of MDCK cells and undergoes cycles of asexual and sexual development. The pace of development in MDCK cells appears to be similar to that described with in vivo infections [2] and provides access to large numbers of meronts and merozoites. Based on our comparisons of 15 different cell lines (including the lines described above), we believe that the MDCK cell line is an excellent host cell for the culture of *C. parvum*. The MDCK cells, like intestinal epithelial cells, are highly polarized when grown to confluency, with distinct apical and basolateral regions as well as a microvillus border [5]. These properties of MDCK cells may contribute to their utility as a host cell for *C. parvum* invasion and development.

We used an enriched medium (RPMI 1640), reduced conditions and low oxygen tension, and multiple additives for the culture of *C. parvum*. The necessity for each additive has not been definitely established. However, the addition of insulin and rabbit bile appeared to increase the number of intracellular parasites and to accelerate their development compared to cultures lacking these additives. The omission of serum from the Cp culture medium had no effect on the number or development of intracellular parasites for the first 24 h, but subsequent development was inhibited.

One advantage of the in vitro culture of *C. parvum* with MDCK cells is that it provides access to large numbers of trophozoites and meronts free from other microorganisms that may contaminate *C. parvum* isolated from the intestines of infected animals. For example, we have successfully used infected MDCK cells as a source of antigen for the production of monoclonal antibodies reactive with intracellular meronts. This culture system also provides a convenient method for evaluating the viability of oocysts, the drug sensitivity of different life cycle stages, and the biology of intracellular development. Unfortunately, however, we have not observed sporogony or long-term propagation and multiplication of *C. parvum* with MDCK cells, suggesting that additional manipulations of the culture system may be necessary to achieve these goals.

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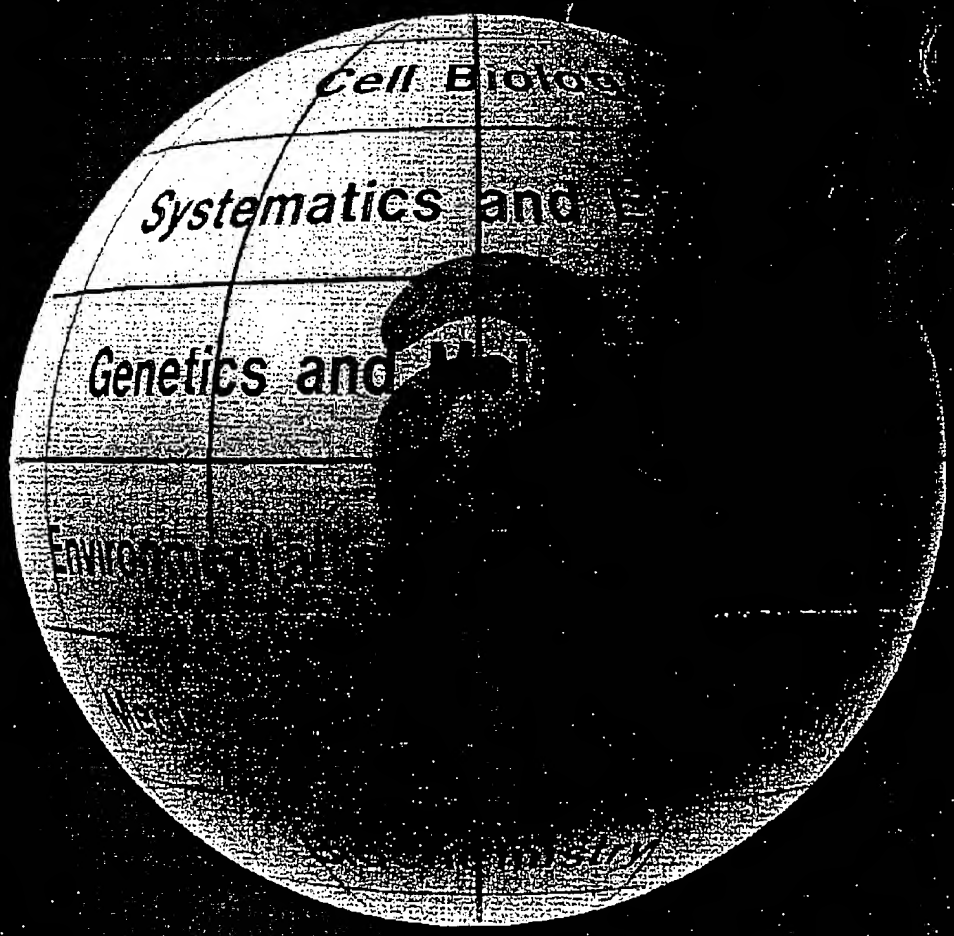

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ORIGINAL PAPER

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Characterization of a monoclonal antibody reacting with antigen-4 domain of gp900 in *Cryptosporidium parvum* invasive stages

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Abstract *Cryptosporidium parvum* (Protozoa, Apicomplexa) infects the apical surface of intestinal epithelial cells, where it grows and divides within a membrane-bound parasitophorous vacuole. gp900, an abundant glycoprotein of *C. parvum* merozoites and sporozoites, is localized in micronemes and at the surface of invasive stages and participates in the invasion process. Here, we describe a new monoclonal antibody (mAb) against gp900. As shown by immunofluorescence of excysted parasites and immunoelectron microscopy of infected tissues, the mAb reacted with micronemes present in the apical pole of invasive stages. In immunoprecipitation experiments, the mAb was shown to react with a high molecular weight antigen co-migrating with gp900. Finally, three reactive clones were selected upon screening of a *C. parvum* genomic expression library with the mAb; and sequencing of the insert from one of them showed a 596 bp sequence identical to the DNA region encoding a domain of gp900 identified as antigen 4.

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Introduction

Cryptosporidium parvum is an Apicomplexan protozoan that develops in the brush border of epithelial cells in the digestive tract of mammals and causes chronic diarrhea in immunocompromized humans, especially in HIV-infected individuals or in malnourished children. The parasite may also cause acute diarrhea in normal adults and children (Current and Garcia 1991; Guerrant 1997; Agnew et al. 1998). Moreover, *C. parvum* is recognized as one of the most ubiquitous and difficult to control agents of water-borne diarrhea (Smith and Rose 1998).

Invasion of intestinal epithelial cells by *C. parvum* sporozoites and merozoites is a critical step for initiation of the infectious cycle. Like other members of the phylum Apicomplexa, such as *Plasmodium* spp. and *Toxoplasma gondii*, invasive *C. parvum* zoite stages possess a highly differentiated complex of secretory apical organelles, the micronemes, rhoptries, and dense granules, whose contents are sequentially released during the events of attachment, invasion, and establishment of the parasitophorous vacuole (PV; Joiner and Dubremetz 1993; Dubremetz et al. 1998). Invasion occurs by zoite attachment to the enterocyte, followed by discharge of the microneme and rhoptry contents, which induce fusion of the enterocyte microvillar membranes over the parasite pellicle. This encloses the parasite in an intracellular, but extracytoplasmic PV that remains in the brush border, at the surface of the enterocyte (Current and Reese 1986; Marcial and Madara 1986).

To examine the molecular basis of these *C. parvum*-enterocyte interactions, we produced monoclonal antibodies (mAbs) that recognized antigens within the zoite apical organelles; and we used them to characterize the identity and fate of cognate antigens during epithelial cell invasion and intracellular development (Bonnin et al. 1991, 1993, 1995). In this paper, we describe mAb IRM that recognizes a *C. parvum* microneme antigen and demonstrate that this mAb reacts with gp900, an

abundant mucin-like glycoprotein previously shown to be involved in host-cell invasion (Barnes et al. 1998).

Materials and methods

Hybridomas secreting mAb IRM were produced and selected in a series of experiments that were described previously (Bonnin et al. 1993, 1995). The IgG isotype of mAb IRM was determined by an immunoenzymatic assay (Behring Diagnostics). For immunoelectron microscopy, small pieces of terminal ileum were obtained from an immunosuppressed rat experimentally infected with a lamb isolate of the parasite. Processing of tissue samples and immunolocalization were performed as described previously (Bonnin et al. 1993, 1995). For immunoprecipitation, proteins from 1×10^7 oocysts/100 µl were diluted with nine volumes of hybridoma culture supernatants from either mAb IRM or mAb 10C6, a previously described anti-gp900 mAb (Petersen et al. 1992; Barnes et al. 1998), protease inhibitors (Doyle et al. 1993) and 1% Triton X-100. After 1 h incubation at room temperature, protein A Sepharose was added to the samples and incubated for 2 h at 37 °C to ensure

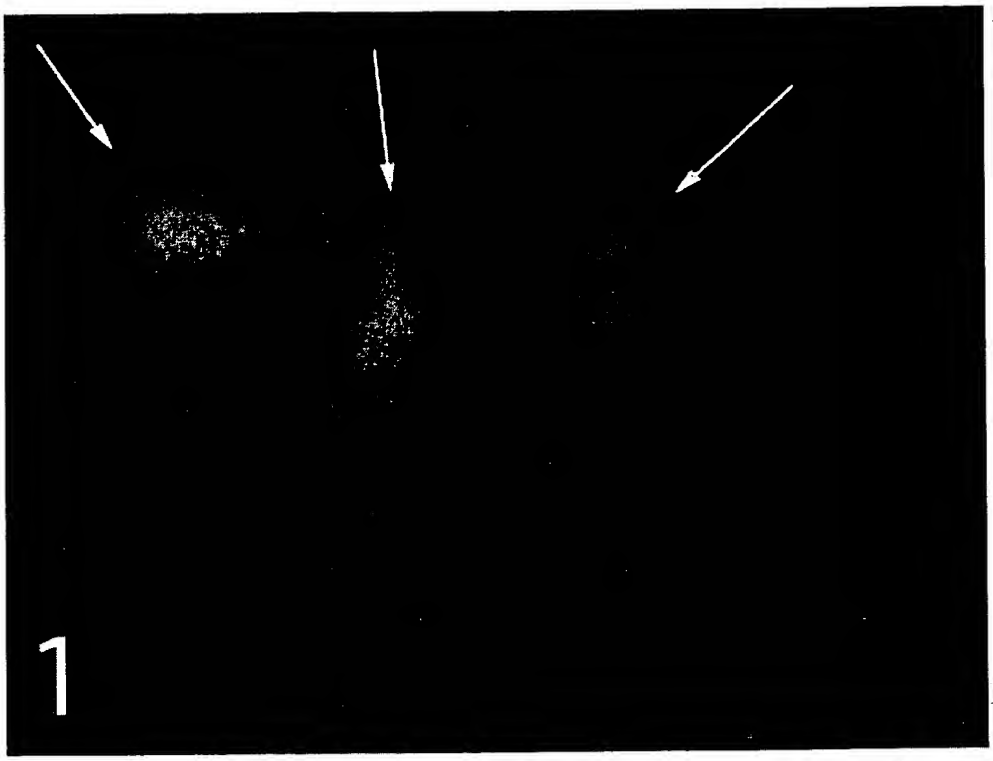

Fig. 1 Immunofluorescence of air-dried, acetone-fixed, excysted sporozoites of *Cryptosporidium parvum* labelled with mAb IRM, followed by a fluorescein-isothiocyanate-conjugated secondary antibody. Evans blue counterstaining. *White arrows* point to the IRM antigen at the apical pole of the parasite

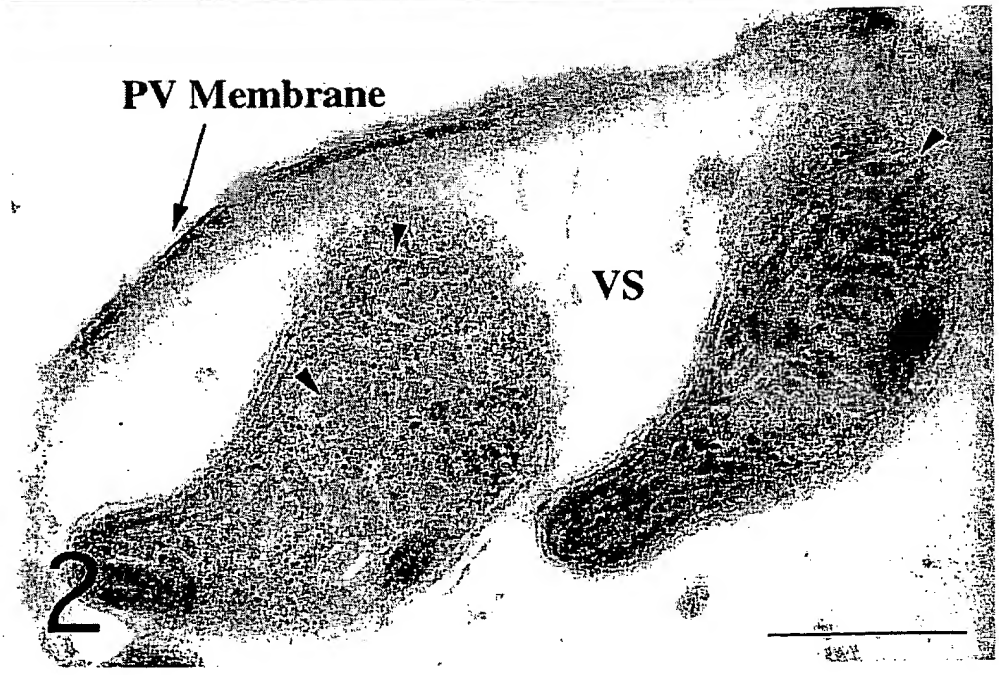

Fig. 2 Immunogold electron microscopy showing reactivity of mAb IRM with a developing meront of *C. parvum* in infected intestinal tissue. Infected tissues were embedded in LR White (London Resin Co.) and thin sections were labelled with mAb IRM, followed by rabbit anti-mouse immunoglobulin serum (Tago) and 8 nm protein A-coated gold beads. The gold particles (*arrowheads*) are clustered in the microneme region of the two merozoites shown in this field. *PV* Parasitophorous vacuole, *VS* vacuolar space. *Scale bar* 0.5 µm

immobilization of the immune complexes. Immunoprecipitated proteins were solubilized by boiling for 5 min in sample buffer; and proteins from approximately 10^7 oocysts/slot were separated by a 5–15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gradient and transferred to nitrocellulose. The membrane was cut into two pieces that were incubated with either mAb IRM or polyclonal serum against gp900 and were revealed using anti-mouse IgG conjugated with alkaline phosphatase and colorimetric development with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate. In order to clone the genomic DNA region encoding the IRM antigen, we screened a *Cryptosporidium parvum* λgt11 genomic DNA expression library (Kim et al. 1992) with hybridoma culture supernatant containing mAb IRM. Three clones were identified in a screen of 10^6 plaque-forming units. They were purified and the clone IRM 15 was sequenced by methods employed previously (Barnes et al. 1998).

Results and Discussion

In Apicomplexan protozoans such as *Plasmodium* spp., *Toxoplasma gondii*, and *Eimeria tenella*, micronemes have been shown to contain molecules used for recognition of, adhesion to, and invasion of host-cells, and for zoite motility (Dubremetz et al. 1998). In *Cryptosporidium parvum*, two microneme proteins have been identified to date, the thrombospondin-related adhesive protein of *Cryptosporidium* 1 (TRAP-C1; Spano et al. 1998), which shares structural features with other microneme molecules such as the TRAP molecules of *Plasmodium* spp. (Robson et al. 1988), the MIC 2 protein of *T. gondii* (Wan et al. 1997), the Etp 100 protein of *E. tenella* (Tomley et al. 1991), and gp900, a highly glycosylated mucin-like protein, which is the target of antibodies that inhibit the invasion and intracellular development of *C. parvum* in vitro (Barnes et al. 1998).

Here, we characterize a mouse IgG mAb that reacts with the micronemes of *C. parvum* invasive stages. The apical immunofluorescence observed with mAb IRM (Fig. 1) was identical to that previously observed using other mAbs that recognize *C. parvum* micronemes (Bonnin et al. 1991, 1993). When mAb IRM was assayed by immunoelectron microscopy on *C. parvum*-infected tissues, strong and specific labelling of micronemes was observed (Fig. 2). No labeling of rhoptries or dense granules was observed, nor was any surface-labelling of sporozoites or merozoites detected. Moreover, no gold particles were observed in the PV or over the vacuolar wall, and no IRM antigen was detected in the host-cell cytoplasm.

The characterization of the antigenic molecules recognized by mAb IRM was achieved by immunoprecipitation of *Cryptosporidium* antigens with IRM or with anti-gp900 mAb 10C6, followed by immunoblotting with IRM or polyclonal antibodies against gp900 (Fig. 3). These experiments showed that mAb IRM precipitated and reacted with several antigenic species, including a high molecular weight antigen that co-migrated with gp900. IRM also reacted with the same molecule in immunoprecipitates obtained using the anti-gp900 mAb 10C6. Taken together, these data suggest that IRM reacts with an epitope of gp900.

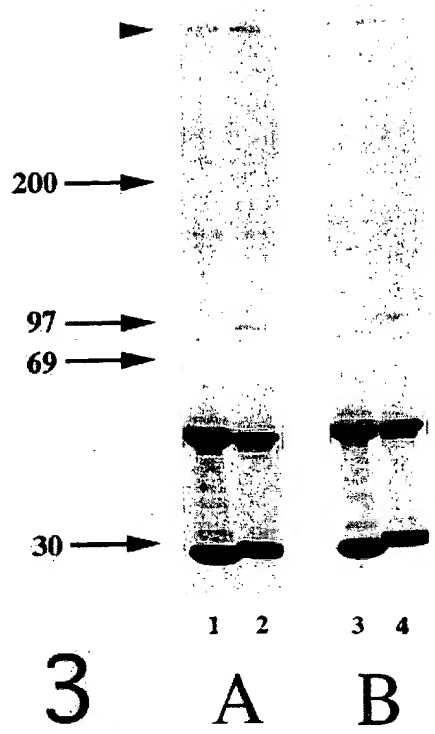

Fig. 3 Immunoprecipitation of *C. parvum* antigens showing the reactivity of mAb IRM with gp900. The immunoprecipitation step was done with mAb IRM (*lanes 1, 3*) or mAb 10C6 (*lanes 2 4*). Western blots were probed with a polyclonal antibody against gp900 (*panel A*) or mAb IRM (*panel B*) and developed with anti-mouse IgG conjugated with alkaline phosphatase, with colorimetric development. *Arrows* refer to molecular weight markers in kilodaltons, while the *arrowhead* corresponds to the expected molecular weight for the gp900 antigen. Reactive bands at about 30 kDa and 50 kDa correspond to the light and heavy chains of the antibodies used for immunoprecipitation, which are recognized by the secondary anti-IgG antibody conjugate used to develop the Western blot

When immunoblotted *C. parvum* antigen was oxidized with periodate under conditions that ablate the antigenicity of carbohydrate epitopes (Bonnin et al. 1991, 1993, 1995), the profile of mAb IRM reactivity was not altered (data not shown), suggesting that IRM binds a protein epitope. The mAb IRM was thus used to screen a genomic DNA *C. parvum* expression library; and three reactive clones were selected, each containing a 600-bp insert. Sequencing of the insert of IRM clone 15 produced a 596 nucleotide sequence identical to the DNA sequence encoding "antigen 4" , one of the previously sequenced regions of gp900 (Barnes et al. 1998). Clone IRM 15 contained a single open reading frame whose predicted sequence was identical to the sequence of amino acids 1,050–1,246 of gp900 from the NINC isolate of *C. parvum* (Petersen et al. 1997).

Although previous studies showed that gp900 surrounds the developing merozoite and is ^{125}I surface-labelled in sporozoites (Petersen et al. 1992; Barnes et al. 1998), no surface-labeling of sporozoites or merozoites

occurred with IRM in immunoelectron microscopy experiments performed in the current study. Similarly, no surface labeling of invasive stages was detected in previous immunoelectron microscopy studies using polyclonal anti-gp900 antibodies (Barnes et al. 1998). This discrepancy could be due to the fixation technique used for immunoelectron microscopy; and ultrastructural immunodetection of gp900 in cryosections of frozen samples or tissues processed with different fixation methods will be required to clarify this matter.

In this study, we describe the characterization of IRM, a mAb that recognizes gp900 in the micronemes of *C. parvum*, and show that the epitope recognized by this mAb resides in the "antigen 4" region of the polypeptide backbone of gp900. This new reagent will provide a useful tool for investigating the biological properties of distinct domains of gp900 and the fate of this molecule during the *C. parvum* enterocyte interaction.

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